Salmonella genes required for virulence and stress response characterization of ClpP and RfbM

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Publication date:
2002

Document version
Publisher's PDF, also known as Version of record

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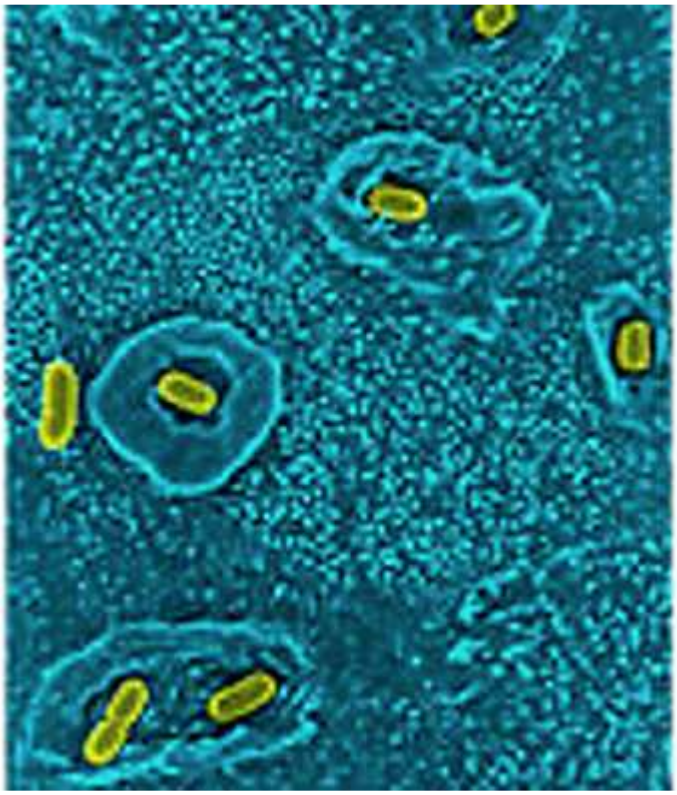

Ph.D. Thesis
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January 2002

Cover:
Salmonella associated with membrane ruffles on the surface of an epithelial cell.
Picture kindly provided by J.E.Galán.

Acknowledgement.

The present work was carried out at The Department of Veterinary Microbiology (IVM) at The Royal Veterinary and Agricultural University, Denmark, The Technical University of Denmark (DTU) and at Department of Microbiology and Immunology at University of South Alabama, USA. The study was supported by The Danish Research Council and The Centre for Advanced Food Studies.

My sincere thanks to my first supervisors, associate Professor Derek J. Brown and Professor John E. Olsen, for their help and guidance, in the first part of this study. A special thanks to associate Professor Hanne Ingmer, for being my supervisor the last part of the project, when Derek left for a new job. I am grateful for Hannes enthusiasm and involvement in the project and for her advises and critics throughout the study. Thanks to Mark Chadfield for introducing me to the world of macrophages.

I owe a special thanks to Professor John W. Foster for his hospitality, when I visited him in Alabama, for all the help I was given and for fruitful discussions. Thanks to all the people in Johns laboratory, for making my stay so pleasant.

The staff at IVM and DTU, for scientific discussions, for qualified technical assistance, but also for cosy Friday afternoons. Sharing office with Kirsten, Marianne and later Dorte and Katja lead to inspiring discussions and a lot of fun. I have very much enjoyed working at IVM.

Finally, a very special thanks to Stine, Anders and Allan for all their help and support.

Copenhagen, January 2002

Line Elnif Thomsen

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Sammendrag.

Salmonella er en vigtig zoonotisk patogen, hvis infektioner af mennesker og dyr har stor økonomisk betydning. *Salmonella*s evne til at inficere en vært kræver forskellige virulens faktorer og en evne til at registrere og respondere på ændringer i omgivelserne. Ved at ændre ekspresionen af forskellige virulens gener som reaktion på forskellige stress påvirkninger, kan *Salmonella* overleve i værten og medføre sygdom. Meget arbejde er blevet lavet i et forsøg på at forstå, hvordan bakterien kan adaptere til de forskellige typer stress den udsættes for i værten. Formålet med mit arbejde var at identificere og karakterisere *Salmonella* gener, som både er involveret i bakteriens respons på stress og som er nødvendige for virulens.

En stor del af den Gram-negative bakteries ydre membran består af lipopolysaccharid (LPS), som spiller en vigtig rolle i *Salmonellas* evne til at forårsage sygdom. O antigen polysaccharid er en komponent i LPS strukturen, som genkendes af værtens immunforsvar. Ved en undersøgelse af *Salmonella enterica* serovar Dublin transposon mutanter, identificerede jeg en *rfbM* mutant, som viste sig at være avirulent i en muse-model. Vækst under forskellige stress betingelser, som høj temperatur, høj salt koncentration, lav pH og tilsætning af hydrogen peroxid, resulterede i langsommere vækst hos mutanten sammenlignet med vildtypen. Ved hjælp af en LPS profil, viste det sig at på trods af mutationen, blev der stadig lavet et normalt O antigen, men ikke i de samme mængder som produceres af vildtypen. Resultaterne indikerer at *rfbM* mutanten har mindre LPS på membranen, og dette påvirker dens mulighed for at overleve i værten.

I mange prokaryote bakterier har man identificeret komponenter af det ATP-afhængige Clp protease kompleks og mange af *clp* generne bliver udtrykt som et respons på stress i cellen. I dette projekt har jeg sekventeret *clpXP* generne fra *Salmonella enterica* serovar Typhimurium og undersøgt betydningen af ClpP for bakterien. Vækst-forsøg viste at ClpP er vigtig for *Salmonellas* evne til at vokse under forskellige stress betingelser, såsom lav pH, høj temperatur og høj saltkoncentration. ClpXP proteasen er involveret i nedbrydningen af RpoS, og da denne er involveret i reguleringen af mere end 50 gener, undersøgte jeg betydningen

af RpoS for den nedsatte vækst. Det viste sig at den nedsatte vækst under stress kan tilskrives både RpoS-afhængige og uafhængige mekanismer. ClpP er også involveret i proteolysen af polypeptider indeholdende puromycyl. Dette indikerer at ClpP fra *S. typhimurium* er involveret i nedbrydningen af misfoldede proteiner, som akkumulerer når bakterier eksponeres for stress. *clpP* mutanten overlevede i makrofager, men viste ingen vækst og dette resulterede i manglende virulens i mus. Resultaterne i dette arbejde viser, at ClpP spiller en vigtig rolle i *S. typhimuriums* evne til at overleve de forskellige stress betingelser som den udsættes for under infektion.

Summary.

Salmonella has long been recognized as an important zoonotic pathogen of worldwide economic significance in human and animals. During infection of its host *Salmonella* uses numerous virulence factors and it is becoming clear that the bacteria have well-developed systems for sensing changes in the environments and respond to these changes by modification of gene expression required for virulence. Much work has been done to further understand the genetics behind virulence and the ability of *Salmonella* to adapt to the stress-full environment in the host. The aim of this work was to identify and characterize genes in *Salmonella*, which are involved in the stress-response in addition to being required for virulence.

Lipopolysaccharide (LPS) is a unique constituent of the bacterial outer membrane of Gram-negative bacteria and a major determinant of virulence in *Salmonella.* The O antigen, an immunogenic repeating oligosaccharide, is a component of the LPS and the genes involved in O antigen synthesis map together at the *rfb* locus. The use of a signature-tagged mutant bank of *Salmonella enterica* serovar Dublin transposon mutants, lead to the identification of an avirulent *rfbM* mutant. The influence of temperature, salinity, low pH and hydrogen peroxide on growth was investigated and I was able to demonstrate that the growth rate under stressful conditions was affected by the transposon present in *rfbM*. An LPS profile revealed that the mutant was able to synthesize the same O antigen as the wild-type, but not the same amount. Thus, it appears that the membrane of the mutant contains less LPS, which affects its ability to survive in the host.

Components of the ATP-dependent Clp protease complex are found in a wide range of prokaryotic cells and they are often expressed as part of the cellular stress response. In this work, the *Salmonella enterica* serovar Typhimurium C5 *clpPX* operon has been sequenced and the function of ClpP investigated. An in-frame deletion of the *clpP* gene was constructed and used to demonstrate that ClpP is important for the ability of *S. typhimurium* to grow under various stressful conditions, such as low pH, elevated temperature and high salt concentrations. Since the stationary phase sigma factor, RpoS is a target of the Clp proteolytic complex, the

effect of the *clpP* deletion was examined in the absence of RpoS and I observed that growth of the *S. typhimurium clpP* mutant is affected through both an RpoS dependent and independent mechanism. ClpP is also involved in proteolysis of puromycyl-containing polypeptides, suggesting that it plays an important role in the degradation of misfolded proteins, which accumulate when *Salmonella* is exposed to stress. Intra-macrophage survival of the *clpP* mutant was strongly restricted, thus resulting in loss of virulence in the mouse model. The results suggest that ClpP plays an important role in the adaptive response of *Salmonella* during the infectious process.

List of Abbreviations.

bp	base pairs
CFU	colony forming units
h	hour(s)
IVET	*in vivo* expression technology
kb	kilo base pair(s)
kDa	kilo Daltons
lcr	large colony revertant
LPS	Lipopolysaccharide
min	minute(s)
scm^+	small colony morphology
SCV	*Salmonella* containing vacuole
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SPI	*Salmonella* Pathogenicity Island
STM	Signature-tagged transposon mutagenesis
2D	two-dimensional
TTSS	type three secretion system
wt	wild-type

Introduction.

1. *Salmonella* interactions with the host.

Salmonella are Gram-negative facultative intracellular pathogens and the bacteria can cause a variety of diseases, ranging from acute gastroenteritis to systemic infections like typhoid fever. *Salmonella* has been recovered from a wide range of animals, including fish, reptiles, birds and mammals, where it inhabit the intestinal tract. Even though *Salmonella* may survive in the environment for longer periods, it is the carrier state, which provides the major source of infection of animals and humans. Excretion results in the contamination of water, food and the environment, but also food containing animal products are often contaminated with the bacteria. Thus, the *Salmonella* infections are primarily a consequence of consumption of contaminated foods or fluids.

Serotyping is used to identify the organism beyond the level of subspecies. The serovars are described on the basis of somatic (O), flagella (H) and capsular (mostly for serovar Typhi)(Vi) antigens (LeMinor and M.Y., 1987). The virulence of specific strains of *Salmonella* in humans and animals is frequently serovar specific. Some serotypes have a restricted host range and are predominantly associated with severe systemic disease in a single host species (Table 1), like *Salmonella enterica* serovar typhi (*S. typhi*) which causes typhoid in humans and *Salmonella enterica* serovar Dublin (*S. dublin*) that is primarily associated with disease in calves and adult cattle, in which abortion often occur (McDonough *et al.*, 1999; Jones, 1992).

Table 1. Examples of host-adapted *Salmonella*.

Host	**Serovar**
Human	*S. typhi, S. paratyphi, S. hirschfeldii, S. sendai*
Cattle	*S. dublin*
Swine	*S. choleraesuis, S.typhisuis*
Poultry	*S. pullorum, S. gallinarum,*
Sheep	*S. abortosovis*
Horse	*S. abortusequi*

Broad-host-range serotypes produce different diseases in different hosts. *Salmonella enterica* serovar Typhimurium (*S. typhimurium*) is a typical broad-host-range pathogen, which is among the serotypes most frequently associated with disease in a number of animal species, including cattle, pigs, horses, poultry, rodents and sheep (Poppe, 2000; Wray, 2000a; 2000b; Fedorka-Cray and Gray, 2000; House, 2000). In addition, *S. typhimurium* often causes gastroenteritis in humans and a disease similar to enteric fever in inbred mice (Bakken, 1950).

The ability of bacterial pathogens to adapt to the environment within the host is essential for their virulence. *Salmonella* encounter a number of different environments both in the nature and during its journey through an infected host. They face a number of challenges that are either due to direct attack by the immune system of the host or results from bacterial entry into tissue sites that affects the growth of the pathogen. During these processes, *Salmonella* have to deal with severe acid, high osmolarity, starvation, changes in temperature and oxidative stress. The bacteria respond to these different situations by expressing virulence factors that are appropriate for each location (Lee, 1994; Finlay and Falkow, 1988). It is therefore not surprising that expression of virulence genes, especially those involved in the initial phase of colonization or those that allow survival in a specific intracellular compartment, exhibit a strong regulatory overlap with stress-regulated genes. Investigation of the physiology of bacterial growth in culture have shown that the bacteria are extremely economical in the choice of the genes that are expressed in response to the environmental conditions (Hengge-Aronis, 1999). The level of expression is highly regulated in response to the specific environment. During colonization of the host the bacteria express the genes required for each step in the infectious cycle in response to the host responses. This efficient regulation of gene expression is required for the bacteria's ability to enter the host and colonize.

S. typhimurium evokes a systemic disease in mice called murine typhoid, which serves as a model for human typhoid fever (Bakken, 1950). Typhoid fever is a systemic disease in which bacteria disseminate to the liver, spleen, bone-marrow, and other organs rich in phagocytic cells. When mice are infected orally, *Salmonella* first have to survive the low pH in the stomach. To cope with this, several acid

survival systems have been shown *in vitro* to allow the bacteria to endure low pH and may help them withstand acid in the stomach (Slauch *et al*., 1997). The bacteria then attach to and invade M-cells and/or epithelial cells of the ileum, gaining access to deeper tissues (Takeuchi, 1967; Jones *et al*., 1994). The invasion of the intestinal epithelium is dependent on several invasion genes, including a specialized secretory apparatus, the type III secretion system (TTSS)(Mills *et al*., 1995). Several components of this TTSS are organized in a structure termed the needle complex (Fig.1, Kubori *et al.*, 1998). This complex spans the bacterial envelope and mediates the interaction with the intestinal epithelium and translocate proteins into the host (Kubori *et al*., 1998).

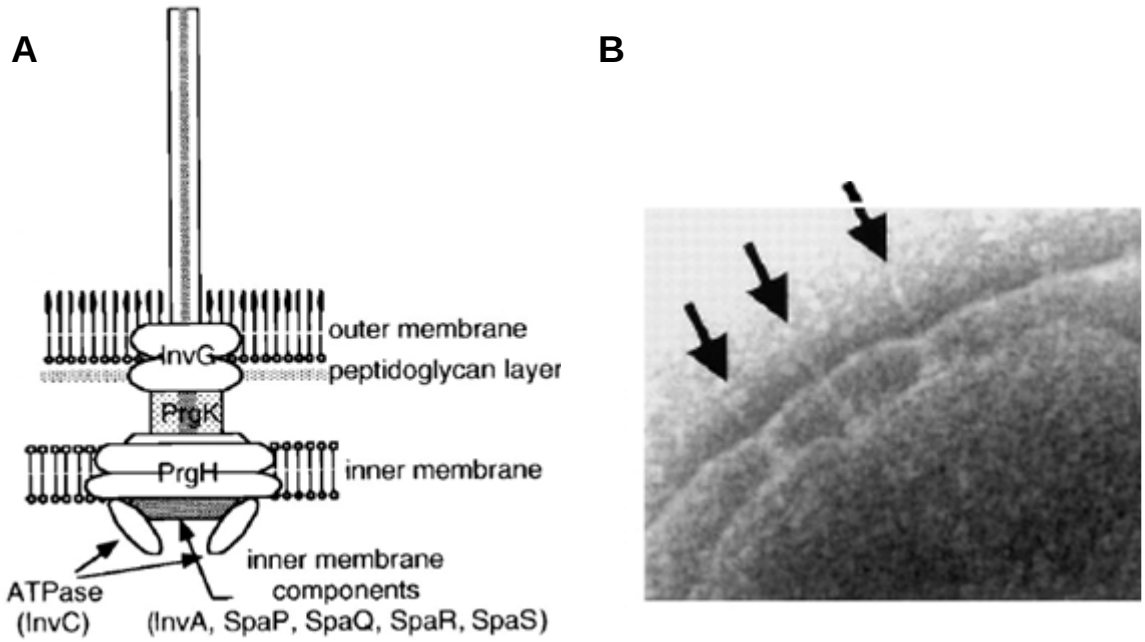


Figure 1. A) Structure of the needle complex. B) Electron micrographs of the needle complex from *S. typhimurium*. Pictures modified from A. Kletzin, personal communication.

The bacteria are believed to trigger a response from the epithelial cell surface shortly after they contact the host cell. *Salmonella* promote the formation of “ruffles” along the membrane of the host cell that engulf the bacteria, which thereby evade the host

immune system (Finlay *et al*., 1991; Garcia-Del Portillo and Finlay, 1994). When *Salmonella* is present in the intestinal lumen, several environmental factors such as low oxygen, high osmolarity and slightly alkaline pH are all conditions that affect the ability of *Salmonella* to enter the cells (Ernst *et al*., 1990; Galan and Curtiss, III, 1990; Lee and Falkow, 1990). Most likely a certain combination of the environmental signals is required to trigger the expression and ensures that the expression is limited to a specific site and time during infection. The invasion genes are usually turned on early in the infection, but are repressed once *Salmonella* is inside the host cell. The bacteria penetrate the intestinal epithelium and reach the Peyer`s patches to colonize underlying mucosal tissue. Here, the bacteria preferentially infect phagocytes wherein the bacteria must quickly adapt to the hostile intracellular environment. Phagocytic cells provide a primary line of defense against invading pathogens and normally result in their rapid destruction. Macrophages have developed an arsenal of oxygen-dependent and –independent mechanisms to effect killing. These include production of toxic oxygen derivatives via the respiratory burst, such as hydrogen peroxide, super oxide anions and hydroxyl radicals. These active metabolites have been shown to cause damage to DNA, RNA, protein and lipids (Hassett and Cohen, 1989). However, *Salmonella* possess antioxidant defenses, which include antioxidant enzymes such as superoxide dismutase and catalase and DNA repair systems (Morgan *et al.*, 1986). *Salmonella* remains in the phagocytes within membrane-bound vacuoles referred to as the *Salmonella*-containing vacuole (SCV) where they resist killing (Finlay and Falkow, 1997), and the survival within the macrophages is essential for *S. typhimurium* virulence (Fields *et al.*, 1986; Leung and Finlay, 1991). *Salmonella* have the capacity to multiply within the vacuoles, which undergoes acidification to pH 4-5 after bacterial uptake (Leung and Finlay, 1991; Rathman *et al*., 1996). Apparently, *Salmonella* requires this acid environment as a cue to initiate intracellular replication and to synthesize factors to allow it to persist in the intracellular environment (Rathman *et al*., 1996). One day after infection, the bacteria can be found in the liver and spleen, predominantly located within phagocytes wherein the bacteria replicate (Hormaeche, 1980). The residence within phagocytes of the liver and spleen is important for a persistent infection, which ultimately leads to the death of the mouse.

2. Bacterial Virulence Factors and Genes.

A greater understanding of the numerous pathogenic characteristics of *Salmonellae* is rapidly emerging through the use of molecular genetic analysis. Many virulence functions are encoded on the *Salmonella* chromosome, but several serovars do also possess a plasmid, which is essential for the ability of *Salmonella* to produce systemic disease (Gulig, 1990). It has been estimated that at least 4% of the genes in *S. typhimurium* encode virulence factors, which are required for fatal infection of mice (Bowe *et al.*, 1998),

2.1 Virulence Plasmids.

Several *Salmonella* species possess a high molecular-weight plasmid ranging from 50kb to 90kb, that is required to cause systemic disease (Gulig and Curtiss, III, 1987; Gulig *et al.*, 1990). Strains cured of this large virulence plasmid remains invasive, but loose their ability to undergo intracellular growth in macrophages (Gulig and Doyle, 1993; Gulig *et al.*, 1998). The genes encoded by the plasmid are also required for bacterial replication in the liver and spleen (Guiney *et al.*, 1995). The plasmid possesses a well-conserved 7.8 kb region encoding five *spv* (*salmonella* plasmid virulence) genes (for a review Gulig *et al*., 1993). The *spv* genes are arranged in two transcriptional units: the first consist of *spvR*, which encodes a positive activator and the latter includes *spvA, spvB, spvC* and *spvD*. SpvR regulates both transcriptional units, whereas the histone-like protein (H-NS), the stationary phase sigma factor (RpoS), the leucine-responsive regulatory protein (Lrp), integration host factor (IHF), and growth phase are involved in the control of *spvR* (Robbe-Saule *et al.*, 1997; Marshall *et al*., 1999; O'Byrne and Dorman, 1994). The exact function of the other Spv proteins is not yet known. Two outer membrane proteins encoded on the virulence plasmid possess resistance to complement activities, but only the *spv* genes on the plasmid are required to confer the virulent phenotype (Gulig *et al*., 1993; Rhen and Sukupolvi, 1988; Vandenbosch *et al.*, 1989).

2.2 Pathogenicity Islands.

A number of important virulence phenotypes in *Salmonella* have been mapped to regions of DNA, the so-called *Salmonella* pathogenicity islands (SPI). These regions appear to have been acquired by horizontal gene transfer (Shea *et al.*, 1996;

Groisman and Ochman, 1993). This is based on the observations that the pathogenicity islands often have a nucleotide composition that is significantly different from that of the host chromosome and by the finding of remnants of insertion sequences, phage or transposon-associated genes, close to these regions. At least five pathogenicity islands and several smaller islets have been identified in *Salmonella enterica* (Wood *et al.*, 1998; Wong *et al.*, 1998; Shea *et al*., 1996; Blanc-Potard and Groisman, 1997).

One of the best characterized *Salmonella* pathogenicity islands, is SPI-1. SPI-1 enable *Salmonella* to invade non-phagocytic cells by inducing membrane “ruffling” and actin cytoskeleton rearrangements accompanied by macropinocytosis, which ultimately directs the internalization of the bacteria into the host cell (Finlay *et al*., 1991; Garcia-Del Portillo and Finlay, 1994). In addition to its role in invasion, SPI-1 is also important for intestinal colonization, activation of cytokine secretion and destruction of M cells in Peyer`s patches (Murray and Lee, 2000; Wallis and Galyov, 2000; Penheiter *et al.*, 1997). Several SPI-1 genes have been identified, including the components of a type three secretion system (TTSS) designated Inv/Spa, which is involved in the delivering of bacterial proteins into eukaryotic host cells. *Salmonella* is unusual since it contains two different TTSS encoded by two different pathogenicity islands. Other SPI-1 genes identified are the effector proteins secreted by the TTSS (Sip and Spt), which induce the otherwise non-phagocytic cells to engulf bacteria, the corresponding chaperones (Ssc) and regulatory components (InvF and HilA) (Bajaj *et al*., 1995; Kaniga *et al*., 1994; 1996; Collazo and Galan, 1997; Zhou *et al*., 1999; Fu and Galan, 1998). The transcription of the secretion system is regulated in response to environmental conditions and bacterial growth state (Ernst *et al*., 1990; Galan and Curtiss, III, 1990; Lee and Falkow, 1990; MacBeth and Lee, 1993). Mutants defective in the Inv/Spa system are attenuated in mice when delivered orally. But they are fully virulent if inoculated intraperitoneally, thereby bypassing the need for invasion of the intestine, suggesting that SPI-1 is not required during systemic infection (Galan and Curtiss, III, 1989).

Another well-described type III system, designated Spi/Ssa is encoded within the SPI-2 pathogenicity island, which is believed to translocate bacterial proteins into the

cytosol of host cells (Shea *et al*., 1996). 32 genes have been identified so far in SPI-2, these include the genes encoding the Spi/Ssa secretion system, secreted effectors of the system (*sse*), their chaperones (*ssc*), a two-component regulatory system (*ssr*) and several genes with unknown function (Ochman *et al.*, 1996; Shea *et al*., 1996; Hensel *et al.*, 1997; 1998). Signals present in the phagosome induce the SPI2 gene expression, and the TTSS of SPI2 contributes to the intracellular survival and replication of *Salmonella* within macrophages and to systemic infection in mice (Deiwick *et al.*, 1999; Hensel *et al*., 1998; Cirillo *et al.*, 1998). Mutants defective in this system are highly attenuated in both orally and intraperitoneally inoculated animals (Cirillo *et al*., 1998; Hensel *et al*., 1998). Phagosomes containing heat killed bacteria or SPI-2 mutants interact with lysosomal compartments and are ultimately degraded (Uchiya *et al.*, 1999; Rathman *et al*., 1997). One of the proteins secreted by the Spi/Ssa system, SpiC, inhibits fusion of the *Salmonella*-containing phagosome with lysosomes and thereby allows bacterial growth inside macrophages (Uchiya *et al*., 1999). In addition, the TTSS encoded at SPI-2 is involved in the initiation of programmed cell death in macrophages (Monack *et al.*, 1996). SPI-2 appears only to be involved in the systemic phases of disease, since SPI-2 mutants had no effect on a diarrhea model in rabbits (Everest *et al.*, 1999). This is consistent with results showing that the SPI-2 genes are not required for virulence in mice in the absence of a phagocytic respiratory burst, indicating that SPI-2 plays a specific role in the ability to avoid oxidase-dependent killing by macrophages (Vazquez-Torres *et al.*, 2000). Figure 2 shows the two virulence strategies, which uses the TTSS encoded on SPI-1 and SPI-2.

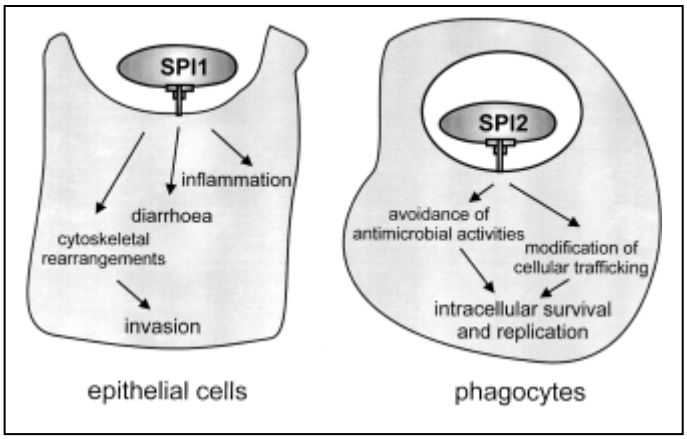


Figure 2. *Salmonella* interaction with host cells. The function of the SPI1-encoded TTSS is required for invasion of host cells and onset of diarrhoeal diseases. In contrast, the function of the SPI2-encoded TTSS appears to be restricted to intracellular *Salmonella*. Figure adapted from Hensel *et al*. (2000).

Further virulence genes are located on the pathogenicity islands SPI-3, which encodes a high affinity Mg^{2+} uptake system required for survival in macrophages (Blanc-Potard and Groisman, 1997). Mutations in SPI-4 confer a macrophage sensitive phenotype (Baumler *et al.*, 1994; Wong *et al*., 1998) and SPI-5 encode proteins required for fluid secretion in the bovine ligated loop model and one of these proteins (SopB) is secreted through the TTSS encoded within SPI-1 (Galyov *et al.*, 1997; Wood *et al*., 1998). *S. typhimurium* needs each virulence gene only at one or at a few specific sites during infection of in the host. The SPI-1 and SPI-2 genes are induced under different conditions using different regulators, which suggest, that once inside a phagocytic host, *S. typhimurium* represses SPI-1 gene expression and turn on genes that are required for growth and survival inside these cells. Mutations in the secretion system of SPI-2 affect the secretion of SPI-1, indicating that there is an interaction between the two type III secretion systems in *Salmonella* (Deiwick *et al.*, 1998). However, the actual mechanisms by which the environmental signals influence gene expression of the appropriate genes are not understood. Regulation of the pathogenicity islands is complex, they encode regulatory proteins controlling the expression of genes within the islands – such as HilA and InvF from SPI-1 and the sensor-kinase system, SsrA-SsrB from SPI-2 (Fig. 3)(Bajaj *et al.*, 1996; Worley *et al*., 2000; Darwin and Miller, 1999a). HilA is also involved in the regulation of genes within SPI-4 and SPI-5 (Ahmer *et al.*, 1999). These regulators are often themselves under the control of global regulators that are encoded outside the *Salmonella* pathogenicity islands. The PhoPQ two-component system plays an important role in the regulation of *Salmonella* virulence genes (Gunn and Miller, 1996; Pegues *et al.*, 1995). PhoPQ regulates genes in response to extracellular cation levels (Garcia *et al*., 1996). When the concentration of cations is low, the sensor kinase, PhoQ, activates the regulator PhoP by phosphorylation. PhoPQ affect the expression of the *spv* genes on the virulence plasmid and genes encoded in SPI-2 positively, whereas the phosphorylated PhoP represses *hilA* after the initial stage of disease is completed (Valdivia and Falkow, 1997; Garcia1996). In contrast to PhoPQ, SirA is an activator of *hilA* (Ahmer *et al*., 1999). Lon negatively regulate the expression of the SPI-1 genes, independent of environmental signals, but possibly through a regulation of *hilA* (Takaya *et al.*, 2002). The global regulator OmpR is also shown to play an important role in the regulation of SPI-2 gene expression by regulating the

expression of *ssrAB* (Lee *et al*., 2000). It seems that the regulation is very complex and that several regulators that measure different parameters may control virulence factors simultaneously, and several regulatory systems can regulate a single virulence factor.

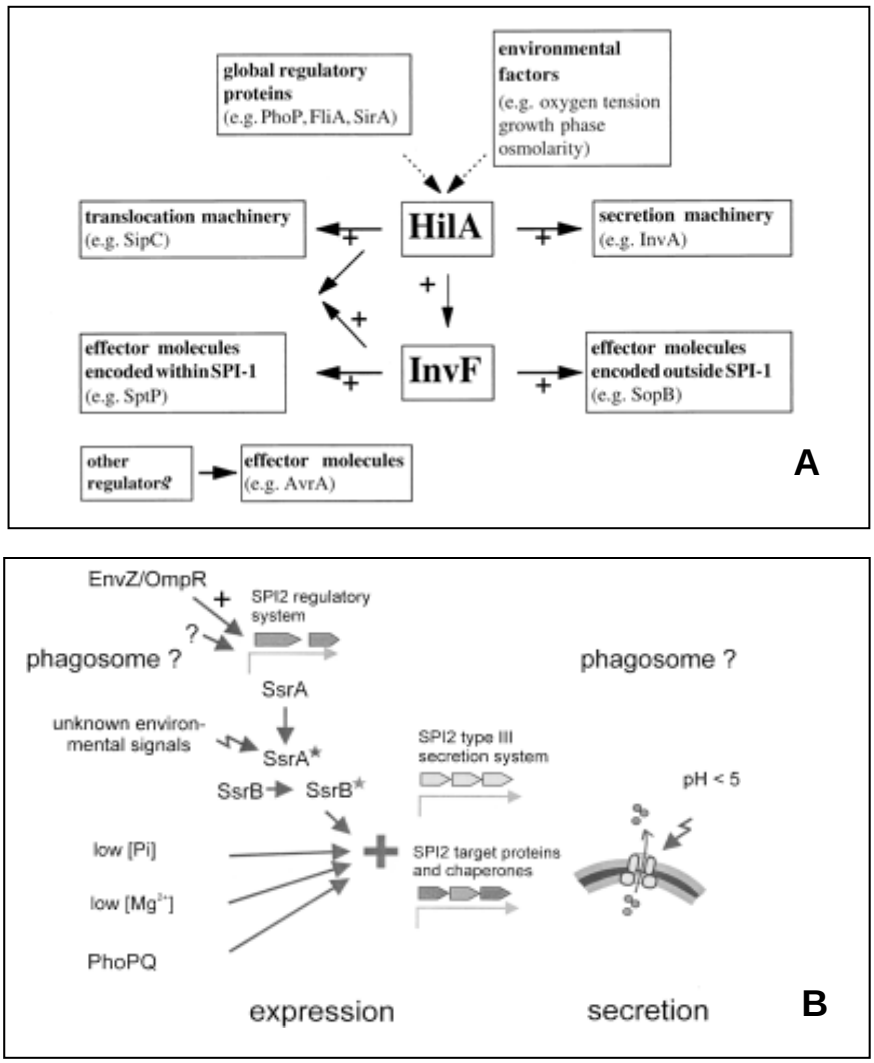


Figure 3. Regulation of SPI-1 and SPI-2 genes. Environmental signals affect the expression of the virulence genes and secretion by TTSS. A) Model for the regulation of SPI-1 virulence gene expression. Figure adapted from Eichelberg and Galan (1999). B) Model for the regulation of SPI-2 virulence gene expression. Figure adapted from Hensel *et al*.(2000).

2.3 LPS.

Lipopolysaccharide (LPS) is an essential component of the outer membrane of Gram-negative bacteria and is a major determinant of virulence in *Salmonella* (Khan *et al.*, 1998; Raetz, 1996). It consists of three regions: (i) the lipid A, which is hydrophobic and forms the outer leaflet of the outer membrane bilayer; (ii) the core polysaccharide, which is further subdivided into the inner and outer core regions; and (iii) the O-chain also called the O-antigen, which is composed of repeating units of sugar residues, which typically comprise 3-6 monosaccharide residues (Fig. 4). The lipid A and the core are highly conserved structurally and genetically within a given genus, but the O-antigen varies considerably with respect to sugar composition, structure, the linkage between sugar residues and antigenicity (Jansson *et al.*, 1981; Raetz, 1996). This variation is used as one of the criteria in the Kauffmann-White scheme for serotyping (Kauffmann, 1966). The members of serogroup A (like *S. paratyphi* A), B (like *S. typhimurium*) and D1 (like *S. dublin*) possess an identical trisaccharide backbone (the O12 antigen), but have paratose (O2-antigen), abequose (O4-antigen) and tyvelose (O9-antigen), respectively, as the immunodominant sugar-branch in their O antigen.

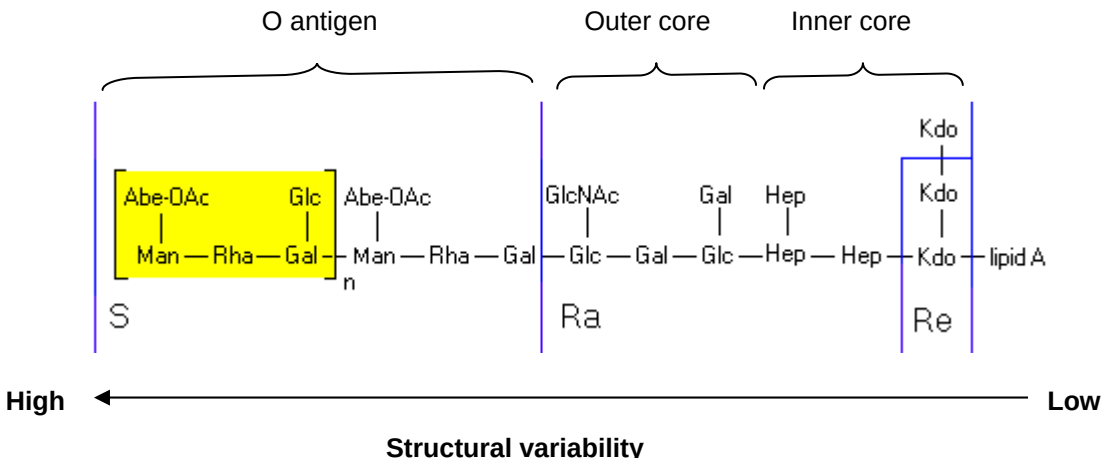


Figure 4. Chemical structure of the *S. typhimurium* LT2 lipopolysaccharide (LPS). The minimal LPS required for growth is the Re chemotype. The Ra chemotype lacks the O antigen and results in rough mutants and S (smooth) denotes the morphology of *Salmonella* with the entire LPS. Abbreviations: Gal, galactose; Man, mannose; Rha, rhamnose; Abe, abesquose; Glc, glucose; GlcNac, N-acetyl-D-glucosamine; Kdo, 3-deoxy-manno-octulosonic acid. Figure adapted from Raetz (1996).

At least 50 genes are required for assembly of LPS (Schnaitman and Klena, 1993). The genes encoding the enzymes that synthesize the sugars and organize them into the polysaccharide side-chains on the LPS are clustered together at the *rfb* locus on the chromosome (Jiang *et al.*, 1991). The O antigens are synthesized separately on a lipid carrier and once completed, O antigen is transferred and covalently linked to the lipid-A-core. *Salmonella* that possess the entire LPS are called smooth (S), because of their colony morphology, while strains that do not contain O antigen often produce irregular edged colonies with a dull surface, which are referred to as rough (Ra), the minimal LPS, which is required for growth is the Re chemotype (Fig.4)(Raetz, 1996). One determination of serum resistance in *S. typhimurium* is the length of the O-antigen side chain. The C3 component of the host complement system deposits on the long LPS O-antigen side chain and the terminal components of the complement system is formed too far from the membrane to be able to disrupt it, so the absence of the O antigen makes the membrane more accessible to the complement and thereby leads to attenuated or abolished virulence in many pathogenic bacteria (Taylor, 1995; Finlay and Falkow, 1988; Joiner *et al.*, 1982). LPS also contributes to disease by virtue of its endotoxic properties, thus, fever, vascular damage in the intestine, and circulatory collapse are considered to be result of LPS produced in the organism (for a review Raetz, 1996).

2.4 Siderophores.

All bacteria require iron for their growth. However, humans and animals have mechanisms for binding iron, which prevent it from being available for infecting microorganisms. Since the availability of iron is affecting the growth of bacteria, the lack of the metal is a major environmental signal to trigger the expression of virulence determinants (Litwin and Calderwood, 1993). Upon entry into deeper tissue pathogens encounter the iron-restricted environment and, similar to many other bacterial pathogens, *Salmonella* have evolved a systems to obtain iron from the limiting environment. A number of iron uptake systems have been identified in *Salmonella*, which include systems that make use of low-molecular-weight compounds, siderophores, which are secreted to the media where they bind the metal ion with high affinity. The siderophore-ion complex is then taken up by the cell via specific outer membrane receptors (Braun, 1991; Baumler *et al.*, 1998).

3. Identification of virulence genes.

Much of the knowledge about virulence determinants of pathogenic bacteria comes from experiments with bacteria grown in culture, where the response of *S. typhimurium* to stress has been investigated at the level of gene expression (Finlay and Falkow, 1997; Foster and Spector, 1995). But various new strategies have been used to identify the genes that are specifically expressed in response to host cells or tissue sites. The expression of these genes is important in causing disease in the animal models.

A simple model system to analyze bacterial gene expression in response to host tissue, is to investigate the interaction between the microorganisms and cultured mammalian cells. It is reasonable to think that additional virulence factors will be identified using mammalian cells, since the environmental conditions inside the host cell are quite different from those found in a laboratory. But the cultured cells do not fully correspond to the conditions found in an animal, since several genes might be expressed in response to humoral, rather than cellular factors. So several techniques use animal models for the identification of virulence genes.

3.1 *In vitro* models.

Fusions to reporter genes like *lacZ* and luciferase have been found useful in the identification of virulence gene expression. By placing the genes that encode assayable proteins like *lacZ* and luciferase under the control of various promoters the transcription can be monitored indirectly. These fusions have been used to identify genes, which results in little expression in culture medium, but are expressed during growth of the bacterium within phagocytic cells (Pollack *et al*., 1986; Klarsfeld *et al*., 1994; Alpuche Aranda *et al.*, 1992; Francis and Gallagher, 1993).

Green fluorescence protein (GFP) has also been found valuable in the identification of virulence genes. GFP reporter constructions have been used to identify promoters that show enhanced activity after bacterial interaction with host cells. Bacteria harboring random transcriptional fusions to the *gfp* gene were pooled and subjected to differential fluorescence induction (DFI). The bacteria were grown in culture media and cells having the lowest fluorescence intensity were collected. This pool was

introduced into a macrophage line and sorted again based on highest fluorescence intensity and greatest promoter strength after the bacteria were internalized (Valdivia and Falkow, 1996; 1997).

Two-dimensional protein gel electrophoresis has also been used to characterize genes induced within host macrophages (Buchmeier and Heffron, 1990). The bacterial proteins were labeled with [^{35}S]-methionine, while macrophage protein synthesis was inhibited by cycloheximide. However this approach can only be used in cultured cells and is unlikely to prove applicable to whole animal models.

3.2 *In vivo* models.

Many limitations associated with the use of *in vitro* models for study of bacterial pathogenesis can be overcome by the use of technologies that detect pathogen gene expression during the course of infection within an animal. But it has to be kept in mind that the use of an animal model of a human disease, may not fully show the bacterial-human interaction.

The *in vivo* expression technology (IVET)(Fig. 5) is a technique used for the identification of bacterial virulence genes specifically induced during infection of a host (Mahan *et al*., 1993). A gene bank is constructed by inserting a random pool of DNA fragments upstream of the promoterless *purA lacZ*. The pool is transformed into a *purA* strain. A wild-type *Salmonella typhimurium* strain is able to replicate within lymphoid glands, in the liver and the spleen, but a *purA* mutant is unable to do so. Therefore replication in these sites requires the *purA* gene to be fused to a promoter that is active within host tissue. To avoid the promoters that are active outside the host, the bacteria that harbor promoters that are active within the host are recovered and plated on lactose MacConkey agar to identify strains having low β-galactosidase activity. These promoters are selectively expressed in the host.

The IVET strategy has now been used in a number of pathogenic species and more than 100 genes have been found, that are induced in mice or macrophages (Mahan *et al*., 1993; Heithoff *et al.*, 1997; Rainey, 1999; Wang *et al.*, 1996a; 1996b). The original IVET strategy has later been modified by replacement of *purA* with *cat*

(Mahan *et al.*, 1995), but this requires that chloramphenicol must be administered and proper concentrations maintained in the infected host tissue. One of the obvious limitations of this technique is, that pathogenicity genes that are expressed at only one stage of infection, or transcribed at low levels, may not be identified via this approach.

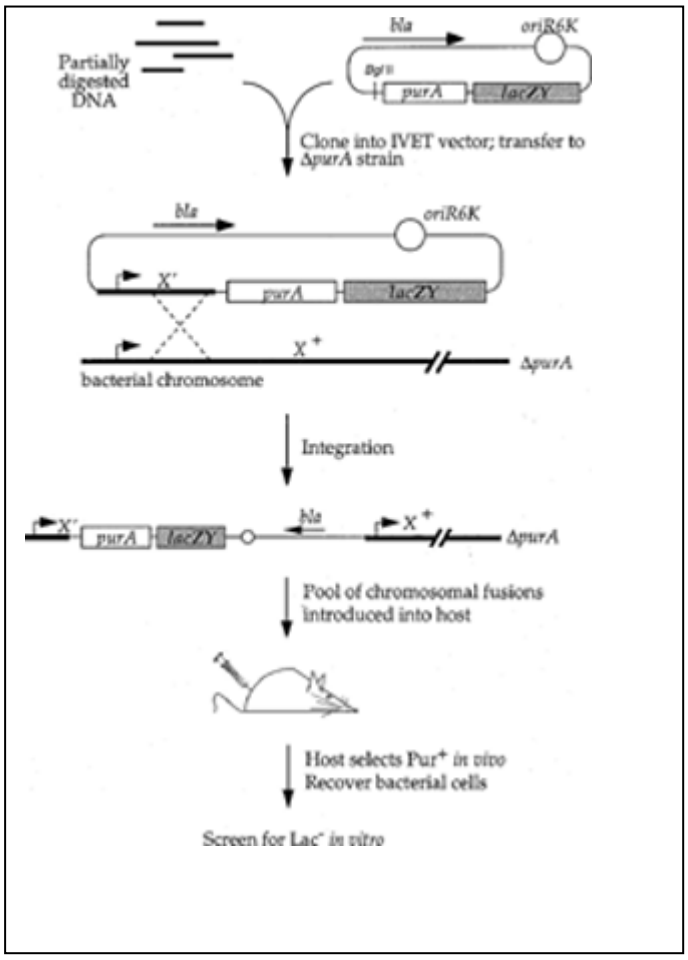


Figure 5. Schematic representation of the IVET selection strategy. See text for details.
Figure adapted from Mahan *et al*. (1993)

Signature-tagged transposon mutagenesis (STM) is a mutation based screening method enabling the identification of virulence genes from a variety of pathogens (Fig. 6; Hensel *et al.*, 1995). By tagging each mutant with a different DNA “signature”, it is possible to screen large numbers of different strains at the same time in the same animal host. The tags were originally designed as short DNA segments containing a 40bp variable central region flanked by invariable arms. Pools of

mutants are inoculated into an appropriate animal host and PCR is used to prepare labeled probes representing the tags present in the inoculum (A and B on Fig.6).

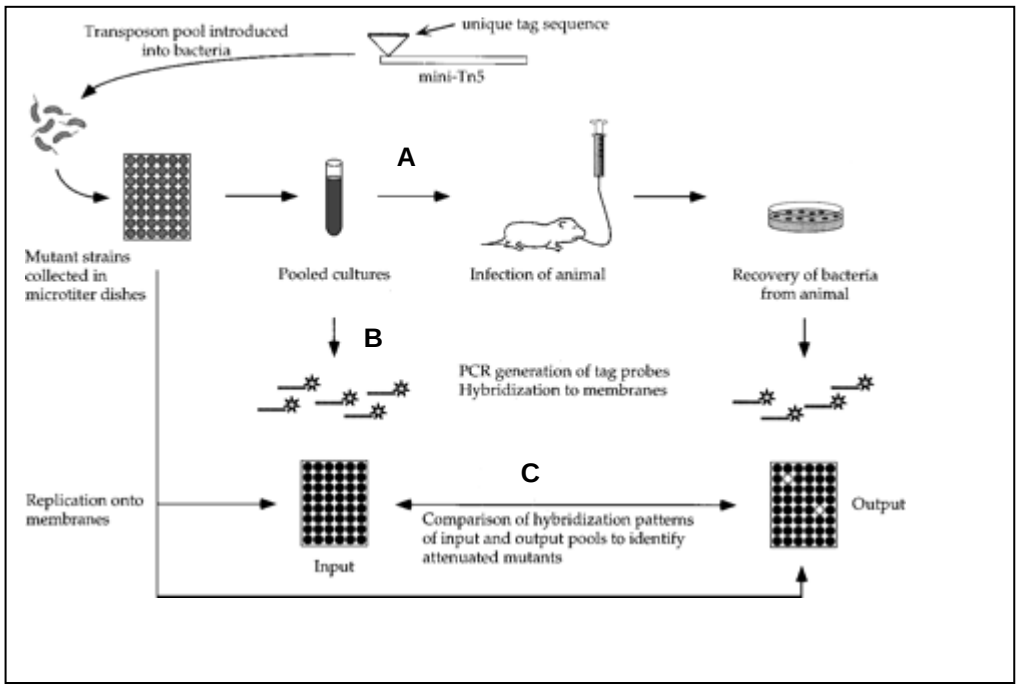


Figure 6. Signature-tagged Transposon Mutagenesis strategy. See text for details. Figure adapted from Hensel *et al*., (1995).

Hybridization of the labeled probes to the tags from the inoculum and the recovered pools from the host permits the identification of mutants that fail to grow *in vivo*, because the tags carried by these mutants will not be present in the recovered pool (C on Fig.6). The limitations of STM include those general to transposon insertion mutagenesis, the randomness of insertions and the polarity effects on operons. It is also possible that some of the transposon insertions have acquired independent spontaneous mutations. Therefore, proof that the interrupted genes are required for virulence will require complementation or the construction of non-polar null mutations. The STM technique has been applied in several microbial pathogens (Hensel *et al*., 1995; Mei *et al.*, 1997; Darwin and Miller, 1999b; Chiang and Mekalanos, 1998; Lehoux *et al*., 1999; Polissi *et al.*, 1998). The use of STM lead to the identification of many known virulence factors as well as other previously

unidentified factors, and it lead to the discovery of the SPI-2 type III secretion system in *Salmonella* (Shea *et al*., 1996). Not all virulence genes are likely to be recovered using STM, since it will only identify genes whose mutant phenotype cannot be trans-complemented by other virulent strains present in the same inoculum. This could include secreted toxins and factors that interfere with an immune response. But the use of STM has recently resulted in the identification of *sseD*, a secreted protein encoded at the SPI-2 (Bispham *et al.*, 2001).

4. Proteases and chaperones.

The ability of *Salmonella* to cope with the hostile conditions in the host is essential for its virulence. During conditions of stress, misfolded and abnormal proteins accumulate and the cell responds to this accumulation by increasing the synthesis of both molecular chaperones and proteases (Goff and Goldberg, 1985). The purpose of these proteins is to either repair or remove the misfolded polypeptides. If synthesis and aggregation of abnormal proteins outpaces degradation and refolding, the cell will accumulate inclusion bodies, which will lead to cell death (Gottesman, 1996). But because of the risk it poses to normal cellular components, intracellular proteolysis must be tightly controlled.

Energy-dependent proteolysis is very important in both prokaryotic and eukaryotic cells to eliminate the abnormal proteins and also to regulate the level of naturally short-lived regulatory proteins (Schirmer *et al.*, 1996; Zhou and Gottesman, 1998; Gottesman and Maurizi, 1992; Goff and Goldberg, 1985). The proteolysis is carried out by multimeric protein complexes such as the proteasomes of eukaryotic and archaeal cells and the ATP-dependent proteases of eubacterial cells. In *E. coli,* several ATP-dependent proteases have been characterized; the Lon-, FtsH- and the Clp-proteases (Porankiewicz *et al*., 1999; Wickner *et al.*, 1994; Gottesman *et al*., 1997). One of the best characterized groups of proteases in *E. coli* is the Clp proteases, which together with Lon, account for up to 80% of the protein degradation in the cell (Goldberg *et al.*, 1994). Another way to deal with misfolded proteins is to promote refolding and proper assembly. This is done by the molecular chaperones, which modulate protein-folding pathways, thereby preventing the formation of incorrectly folded structures. Recent work have shown that components of the Clp

protease, besides the involvement in the proteolytic degradation, also posses chaperone activity (Wickner *et al*., 1994; Singh *et al.*, 2000). The components of the Clp protease are ubiquitous and are found in both prokaryotic and eukaryotic cells (Maurizi *et al.*, 1990a; Gottesman *et al*., 1997). However, their function and targets vary from one organism to another, and several have important roles in stress response and virulence.

4.1 The Clp protease.

The first ATP-requiring protease discovered in *E. coli* was the Lon protease (Chung and Goldberg, 1981). Analysis of *lon* cells revealed that they were only partially deficient in protein degradation, suggesting that other proteins could be involved in the ATP requiring proteolysis (Maurizi, 1987). This lead to the finding of a new ATP-dependent proteolytic system, referred to as protease Clp, caseinolytic protease, since casein was the first known substrate to be degraded (Katayama-Fujimura *et al*., 1987; Katayama *et al.*, 1988). Unlike the Lon protease, which is a homo-oligomer in which both proteolytic and ATPase activity reside, the Clp protease is a large complex that consists of a proteolytic subunit (ClpP or ClpQ) and a regulatory ATPase subunit which confers substrate specificity (Gottesman *et al*., 1997; Squires and Squires, 1992; Wawrzynow *et al*., 1996). *clpP* from *E. coli* encodes a 207 amino acid polypeptide, of which a 14 amino acid leader is rapidly cleaved to yield the mature protein (Maurizi *et al.*, 1990b). Only the mature ClpP protein is proteolytic active, so the cleavage of ClpP leads to activation, which might be a way of regulating the level of active ClpP in response to certain conditions. The crystal structure of ClpP has been determined and ClpP was shown to form a hollow barrel-like structure composed of two rings of seven subunits stacked back-to-back in which the substrates are translocated for proteolytic degradation (Wang *et al*., 1997; Maurizi *et al*., 1990b). ClpQ, a threonine protease, has a similar architecture, except that the symmetry is six fold (Kessel *et al.*, 1996; Rohrwild *et al.*, 1997). ClpP is a serine-type protease with conserved catalytic triads, with the catalytic sites completely compartmentalized inside the barrel. A serine, a histidine and an aspargine residue required for the catalytic activity have been identified (Maurizi *et al*., 1990a; Wang *et al*., 1997). The structure of ClpP is analogous to the 20S proteasomes of eukaryotes and archaebacteria, with multiple active sites residing in

the interior of the multimeric rings (Kessel *et al.*, 1995). Access to the proteolytic chamber appears to be through narrow pores at either end of the chamber (Wang *et al*., 1997). This narrow entrance to the cylinder restrict access to small polypeptides and unfolded proteins and prevents the degradation of normal cellular components. Like Lon, ClpP do not require ATP for the hydrolysis of smaller peptides, but the degradation of larger proteins requires both the ATPase component and ATP hydrolysis (Lugtenberg and Van Alphen, 1983; Wojtkowiak *et al*., 1993).

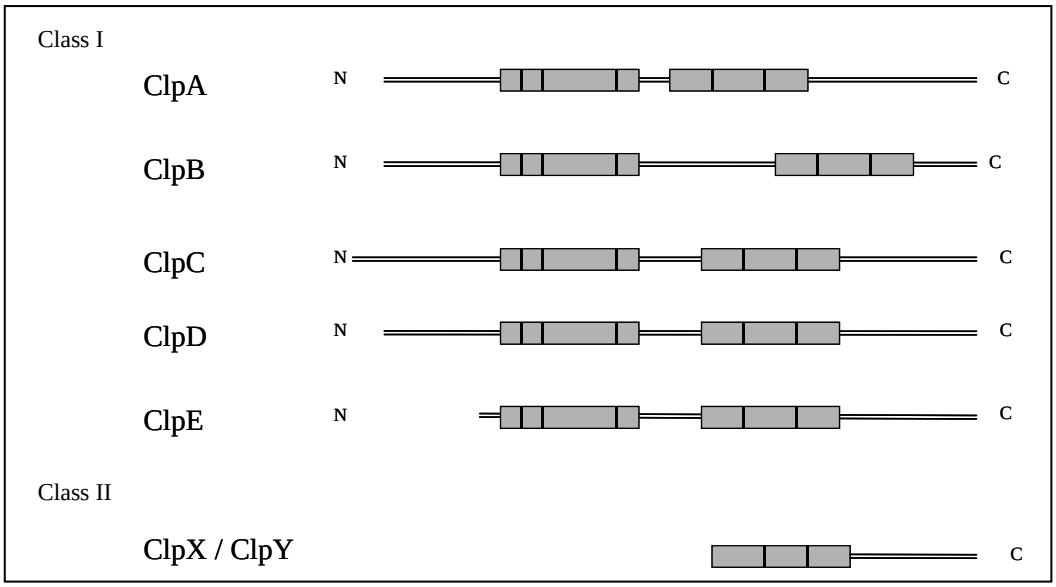
4.2 The Clp ATPases.

The ClpP barrel of *E. coli* can be flanked by one or two hexameric rings of one of the ATPases ClpA or ClpX, which confer substrate specificity to the complex (Kessel *et al*., 1995; Grimaud *et al.*, 1998). ClpA and ClpX can catalyze protein unfolding and are able to function independently of ClpP as molecular chaperones (Wickner *et al*., 1994; Singh *et al*., 2000). The unfolding activity of the ATPases is probably important to promote proteolysis by delivering the unfolded substrate into the active site of ClpP. The ClpB ATPase is another ATPase highly similar to ClpA. This ATPase also function as a molecular chaperone able to resolubilize the protein aggregates that accumulate during heat shock, but apparently it is unable to associate with ClpP (Gottesman *et al.*, 1990; Moczko *et al.*, 1995; Woo *et al.*, 1992).

Homologues to the ClpA, B and X ATPases of *E. coli* have been found in both prokaryotic and eukaryotic cells. This family of ATPases is also known as the Hsp100 family. The ClpP protein itself has no homology to the family of Clp ATPases. Comparison of Clp ATPases from different organisms has revealed several subfamilies, which can be divided in two classes dependent on the number of ATP-binding sites and specific signature sequence motifs (Fig. 7). Class I contains at least five subfamilies, ClpA, ClpB, ClpC, ClpD and ClpE, which all have two highly conserved nucleotide-binding domains (ATP-1 and ATP-2) (Schirmer *et al*., 1996). The ATP-1 and ATP-2 are non-homologous and *in vitro* mutagenesis of ClpA ATP-1 and ATP-2, suggest that the effect of binding of ATP to these two domains have different functions. The binding of ATP to one domain is required for ClpA oligomerization and chaperone activity, whereas ATP binding to the second ATP-binding site is essential for ATP hydrolysis (Pak *et al.*, 1999; Singh and Maurizi,

1994). However, both domains have overlapping functions that are critical for optimal activity (Pak *et al*., 1999). Interestingly, in the case of the yeast homologous, Hsp104, the role of the two nucleotide binding domains are reversed (Schirmer *et al.*, 1998).

A



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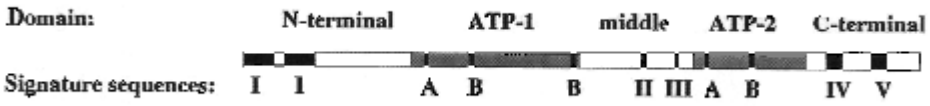


Figure 7. Conserved regions of the Clp ATPase family. (A) The black bars represent the consensus sequences present in a highly conserved nucleotide-binding site. The class I proteins contain a N-terminal and a C-terminal domain, two ATP binding sites and a variably sized middle domain. The class II proteins are shorter in length, containing only one ATP-binding domain. Figure adapted from Wawrzynow *et al*., (1996). (B) The signature sequence motifs are indicated by black boxes and numbers and the ATP-binding domains by ATP-1 and ATP-2 (shaded bars). Figure adapted from Ingmer *et al*., (1999).

The five subfamilies can be distinguished based on the length of the spacer region between the two domains. The middle region of ClpA-type proteins are shorter (~54 amino acids) compared to that of the ClpC- and D-type (~101-118 a.a. residues) and

ClpB-type (~172-207 a.a.) (Squires and Squires, 1992; Schirmer *et al*., 1996). Most members of the ClpC and ClpE family are found in Gram-positive bacteria, cyanobacteria and plants. ClpD is probably unique to higher plants and so far only found in *Arabidopsis thaliana* (Kiyosue *et al*., 1993). ClpX belongs to the Class II proteins, which also include ClpY, the ATPase subunit that interact with ClpQ. These ATPases only have one nucleotide binding site, which is highly homologous to the one responsible for hydrolysis, and a C-terminal domain with two conserved regions (signature sequence IV and V, Figure 7B) (Gottesman *et al.*, 1993; Schirmer *et al*., 1996). ClpY differs from ClpX by the presence of a spacer region within the ATP-binding domain (Gottesman *et al*., 1993).

Besides the ability of ClpA and ClpX to form complex with ClpP, also ClpE and ClpC are likely to be partners of ClpP (Turgay *et al.*, 1998; Nair *et al.*, 1999). Characteristic for the ClpX and ClpE ATPases is a putative zink finger motif, which is important for ATP binding, protein oligomerization and protein-protein interactions (Banecki *et al.*, 2001). This is consistent with the observation that the molecular chaperone DnaJ also contains a zinc-finger motif, which is involved in the binding of misfolded proteins (Banecki *et al.*, 1996). In addition, ClpE has the conserved signature sequence II (Figure 6B), which is also characteristic for the ClpC and ClpD families (Schirmer *et al*., 1996). Although the signature sequence II is characteristic for the ClpC family, ClpC from *Lactococcus lactis* lacks this conserved sequence (Ingmer *et al.*, 1999).

4.3 Substrates for the Clp proteases.

Cleavage of the wrong polypeptides could be damaging or even lethal, so the recognition of the correct substrate is important. *E. coli* ClpP is able to form the active protease in complex with ClpA or ClpX (Katayama-Fujimura *et al*., 1987; Gottesman *et al*., 1993; Wojtkowiak *et al*., 1993) and since the two ATPases have different substrate specificity, different cellular activities can be affected (Wojtkowiak *et al*., 1993; Wickner *et al*., 1994).

Besides the role in the degradation of misfolded proteins, the regulated proteolysis is also important for rapid and precise regulation of the cellular level of regulators. The

ClpAP complex is known to degrade the plasmid origin-binding factor RepA, the regulator of heme biosynthesis, HemA and the MazE protein, which in *E. coli* is a regulator of cell death (Wickner *et al*., 1994; Aizenman *et al*., 1996; Wang *et al*., 1999). A ClpA-lacZ fusion is also a substrate for ClpAP hydrolysis, suggesting that ClpA is involved in the regulation of its own concentration in the cell (Maurizi *et al.*, 1994). ClpXP is known to degrade the bacteriophage origin-binding protein λO, the P1 plasmid Phd protein, the starvation sigma factor RpoS and it is also involved in the turnover of the Mu repressor (Schweder *et al.*, 1996; Gottesman *et al*., 1993; Lehnherr and Yarmolinsky, 1995; Mhammedi-Alaoui *et al.*, 1994). The proteins that are substrates for the degradation by the Clp protease, are in many cases also substrates for the chaperone activity. ClpA, but not ClpX, is able to activate RepA, and ClpX can disassemble MuA-DNA complexes and disaggregate λO, which ClpA is unable to do (Wickner *et al*., 1994; Pak and Wickner, 1997; Wawrzynow *et al.*, 1995; Levchenko *et al*., 1995; Kruklitis *et al*., 1996). The mechanism of substrate selection is still relatively unknown, but recent studies have indicated that the substrate specificity of ClpX and ClpA depends at least in part on the C-terminal sequences of the target proteins (Laachouch *et al.*, 1996). Substitutions in the first ten amino acids in the C-terminal domain of MuA makes the protein resistant to degradation by ClpXP, and additionally, if the ten residues from MuA are fused to another protein, it is possible to convert this protein to a substrate for the ClpXP protease (Levchenko *et al*., 1997). But there is no apparent homology among the C-terminal sequences of the ClpX substrates, suggesting that other signals are important for recognition. For other substrates, HemA, UmuD´ and λO and a LacZ-fusion protein bearing hydrophobic N-terminal amino acids, recognition is through sites in the N-terminal region (Kowarz *et al.*, 1994; Wang *et al*., 1999; Gonciarz-Swiatek *et al.*, 1999; Tobias *et al.*, 1991). There is some overlap in substrate recognition between ClpA and ClpX, since the two proteases ClpAP and ClpXP are able to recognize and degrade SrrA tagged polypeptides, although ClpXP is largely responsible for this degradation *in vivo* (Gottesman *et al.*, 1998; Weber-Ban *et al.*, 1999; Kim *et al.*, 2000). When translation in *E. coli* is interrupted, the peptide can be modified by the addition of an 11 residue C-terminal peptide tail to the nascent chain, a modification mediated by SsrA RNA. The ribosome is able to switch from the damaged mRNA to the SsrA RNA (Keiler *et al*., 1996). This is a way for the cell to tag

proteins, which are being synthesized by damaged mRNA, for recognition and degradation by C-terminal specific proteases. A similar tagging system is also known in eukaryotic cells, where the ubiquitin system tags and targets the abnormal proteins for degradation (Ciechanover, 1994; Hochstrasser, 1995). Other proteases do also seem to have overlapping substrates, ClpQY, ClpAP, FtsH and Lon all seem capable of degrading the heat shock sigma factor (σ^{32}) (Kanemori *et al.*, 1997). The cell division inhibitor SulA is also recognized and degraded by both ClpYQ and Lon (Wu *et al*., 1999). The overlapping specificities of the proteases may provide an effective way of ensuring a rapid response to an accumulation of abnormal proteins.

SspB, a ribosome-associated protein, binds SsrA-tagged proteins and enhances the efficiency with which they are recognized by ClpXP (Levchenko *et al.*, 2000). ClpXP also requires a substrate-binding protein (RssB) in order to recognize and degrade RpoS (Muffler *et al.*, 1996). The existence of such substrate-binding proteins that enhances recognition by the Clp protease may explain the lack of recognition signal on the substrate.

In the attempt to understand the features, which are recognized on the protein substrate, it is also interesting to understand how the protease mediates this recognition. The Clp and Lon proteases themselves contain homologous sequences, called sensor- and substrate-discrimination- or SSD- domains, which mediate recognition and binding to protein substrates (Fig. 8)(Smith *et al*., 1999).

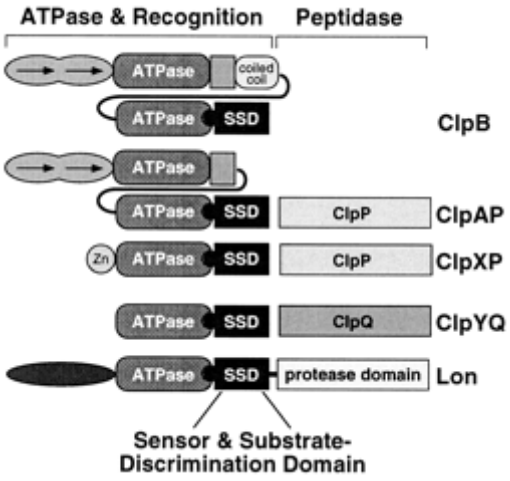


Figure 8. Domain structure of Lon and Clp proteins. The domain suggested to recognize substrates are show as SSD. Figure adapted from Smith *et al*., (1999).

Since the ATPases are able to function both as proteases and as chaperones, it would be expected that there is a mechanism, which decides whether polypeptide chains are repaired or cleaved. A model proposed to account for this switch between degradation and refolding, states that the Clp ATPases recognize and binds sequences or structures, which in the mature protein are masked (Fig.9). According to this model, the misfolded protein can bind both ClpA and the ClpAP complex. The binding of ClpA to the substrate requires ATP-binding but not hydrolysis (Hoskins *et al.*, 2000). Upon hydrolysis, ClpA mediates unfolding of the bound substrate, allowing it to be refolded or translocated into ClpP. ATP is also found to be required for the translocation of the unfolded protein into ClpP (Weber-Ban *et al*., 1999; Singh *et al*., 2000). No steps in the degradation pathway require separation of the ClpAP complex to a degree that allows exchange of subunits, and once assembled, it remains as an intact complex through multiple rounds of substrate turnover (Singh *et al*., 1999). This suggests that ATP hydrolysis may be critical for a conformational change of ClpA that enable unfolding and translocation (Ishikawa *et al.*, 2001). Both chaperone and protease activity can occur concurrently in the ClpAP complex (Pak *et al*., 1999).

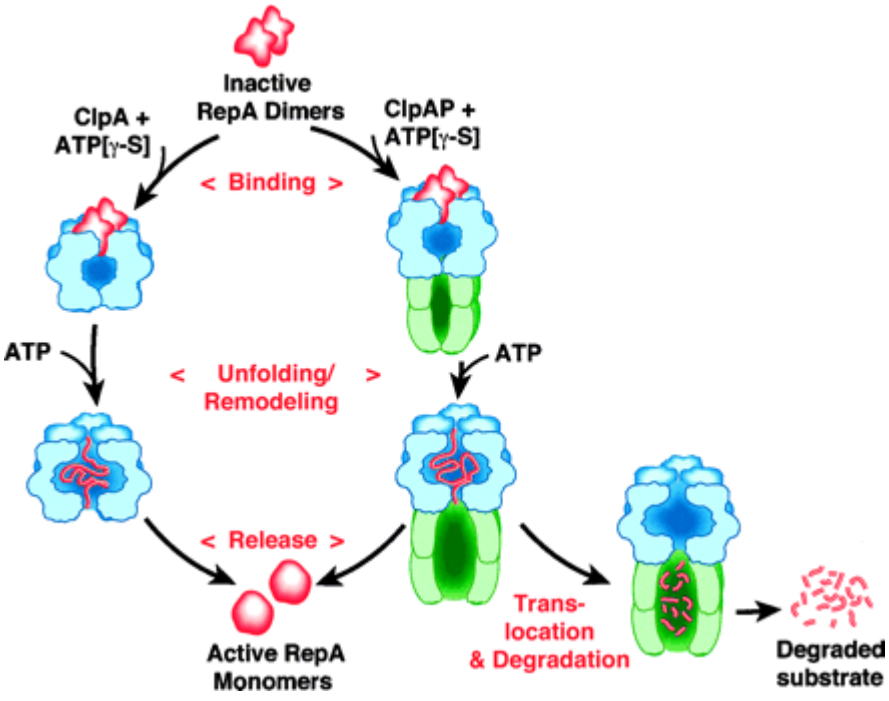


Figure 9. Model of the pathway of degradation or refolding by ClpAP/ClpXP
Figure adapted from Pak *et al*., (1999).

Consistent with this model is the observations of the order of assembly of the ClpA, ClpP and RepA. ClpA-ClpP-RepA assemble by the binding of RepA to the ClpA-ClpP complex or ClpP binding the ClpA-RepA complex. Both of these complexes have been isolated (Wickner *et al*., 1994; Pak and Wickner, 1997; Hoskins *et al.*, 1998). Chaperone activity of ClpA can function in the proteolytic ClpAP complex as well as in free ClpA (Pak *et al*., 1999). Several results indicate that a very similar mechanism for degradation by ClpXP is likely (Singh *et al*., 2000; Jones *et al*., 1998). Even though results with ClpAP and RepA do support this model, we do still not know how the decision for either degradation or refolding is made. A possibility is that the relative concentrations of ClpA/ClpX and ClpAP/ClpXP as well as the relative kinetic parameters for refolding or degradation of a substrate are critical for the fate of a substrate.

4.4 Stress response.

The expression of many of the Clp proteins are induced under conditions of stress and the signal responsible for the induction is believed to be an increase in the cellular concentration of misfolded proteins (Frees and Ingmer, 1999; Msadek *et al.*, 1998; Kroh and Simon, 1990; Volker *et al.*, 1994; for a review Gottesman, 1996). In several bacteria, mutations in the Clp ATPases have a severe effect on growth under stress conditions (Ingmer *et al*., 1999; Nair *et al*., 1999; Kruger *et al*., 1994; Rouquette *et al.*, 1998; Eriksson and Clarke, 1996; Allan *et al*., 1998; Squires *et al.*, 1991). The proteolytic part of the Clp protease seems to be particular important for Gram-positive bacteria. *clpP* mutants of *B. subtilis, L. monocytogenes* and *L. lactis* are more sensitive to stress compared to wildtype (Gaillot *et al.*, 2000; Frees and Ingmer, 1999; Gerth *et al.*, 1998). This is in contrast to results from an *E. coli clpP* mutant, where no change in phenotype, compared to wildtype, is observed (Maurizi *et al*., 1990b).

The *E. coli* sigma factor σ^{32} is involved in the regulation of genes in response to stress and most members of the σ^{32} (RpoH) regulon have been classified as either molecular chaperones or ATP-dependent proteases (Gross, 1996; Parsell and Sauer, 1989; Kanemori *et al*., 1994). This regulon include the Clp ATPase *clpB* and also the *clpPX* and *clpQY* operons, which clearly suggest that these proteins are

indeed important for the survival of the cells during stress. In Gram-positive bacteria, which lack σ^{32}, ClpP and the Clp ATPases are part of the CtsR regulon (Derre *et al*., 1999). CtsR act as a repressor in both *B. subtilis* and *L. lactis* by binding to the promoter region (Derre *et al*., 1999; Varmanen *et al*., 2000).

Bacteria are constantly regulating gene expression according to changes in the environment and two of the regulators are the alternative sigma factors σ^{32} (RpoH) and σ^{S} (RpoS). When *E. coli* cells are exposed to high temperature, there is an increase in the level of σ^{32}, which directs RNA polymerase to transcribe the heat shock genes (Gross, 1996). The increase in the level of σ^{32} depends both on increased synthesis of the protein but also on a change in stabilization (Straus *et al*., 1987). It appears that several proteases, including ClpQY, ClpAP, FtsH and Lon, are involved in the regulation of this sigma factor (Kanemori *et al*., 1997). It seems to be a complex regulation, since many different proteases are involved in the degradation of σ^{32} and some of these are also regulated by the level of σ^{32}. RpoS plays an important role in the survival of *E. coli* and *S. typhimurium* following sudden encounters with a variety of stress conditions (Paesold and Krause, 1999; Hengge-Aronis, 2000). Under exponential growth when the nutrients are readily available, the level of RpoS is low. The level of RpoS increases in stationary phase or during stress, and triggers the induction of more than 50 genes important for survival under unfavorable environmental conditions (Hengge-Aronis, 1996; 2000; Loewen and Hengge-Aronis, 1994; Schweder *et al*., 1996). The amount of RpoS is regulated at multiple levels, which include transcription, translation and proteolysis of σ^{S} (for a review Hengge-Aronis, 2000). ClpXP rapidly degrades the RpoS during exponential growth in order to prevent the induction of the stationary phase genes (Schweder *et al*., 1996; Zhou and Gottesman, 1998). Recently, a possible two-component response regulator from *E. coli*, RssB, or in *S. typhimurium*, MviA, was found to be involved in the RpoS degradation (Pratt and Silhavy, 1996; Bearson *et al.*, 1996; Muffler *et al*., 1996). MviA/RssB is possibly sensing environmental signals via phosphorylation and act by binding RpoS in a 1:1 complex (Fig.10). From this complex, RpoS is delivered to ClpXP where it is unfolded and degraded and MviA/RrsB is released from the proteolytic complex to reinitiate another round of σ^{S}

binding (Zhou *et al.*, 2001; Becker *et al*., 1999; Moreno *et al.*, 2000). When the cell enter stationary phase, the half-life of σ^S increases more than 10 fold and it appears that RpoS acquires greater resistance to degradation by ClpXP (Bearson *et al*., 1996; Lange and Hengge-Aronis, 1994; Webb *et al.*, 1999).

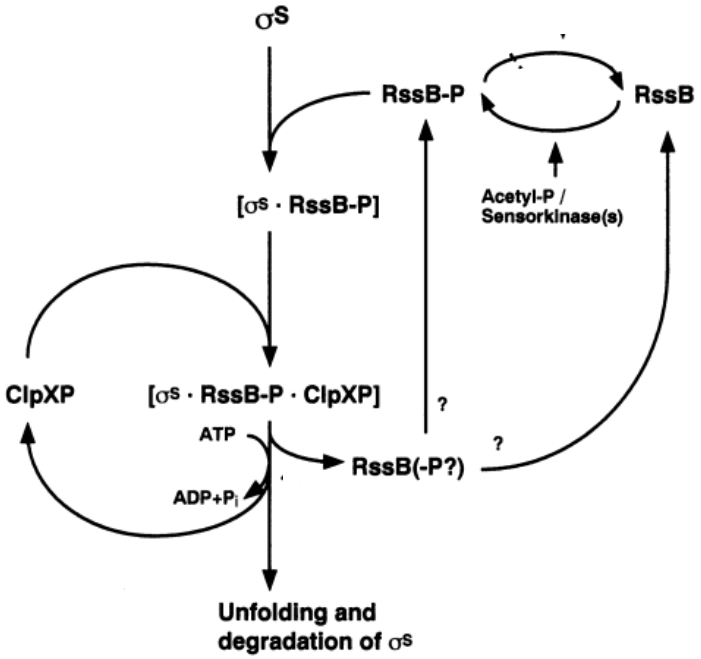


Figure 10. Role of RssB in the initiation of σ^S proteolysis. Figure adapted from Klauck *et al*. (2001)

This could partly be due to changes in the phosphorylation of RssB/MviA, but the level of ClpP and ClpX could also be important. When the cell encounters stress, the ratio of ClpP protein to the ClpX protein increases, probably due to premature transcription termination (Li *et al*., 2000). The amount of ClpA increases under the same conditions (Li *et al*., 2000), making it more likely to complex with ClpP, decreasing the level of the ClpXP protease. ClpXP plays an important role in the regulation of the expression of growth-related genes, but despite the stress inducibility and importance for RpoS degradation, an *E. coli clpP* mutant is dispensable under all growth conditions tested, presumably because alternative proteases compensate for its absence (Maurizi *et al*., 1990b).

4.5 Clp proteases and virulence.

Virulent microorganisms survives and multiplies within host cells, where they encounter hostile conditions, such as nutrient limitation, temperature changes, variations in osmolarity and pH, in addition to the host defense. These conditions induce the cell stress response and the implication of general stress proteins in virulence has already been described for many pathogens. The Clp proteases are widely conserved in bacteria and important for survival under stress and both the proteolytic- and the ATPase-components are found to play important roles in the virulence of many pathogens. Several Clp protease components important for virulence are listed in Table 2, some of which have been identified using Signature Tagged Mutagenesis (Hensel *et al*., 1995; Mei *et al*., 1997; Polissi *et al*., 1998; Coulter *et al.*, 1998).

Table 2. Clp proteins required for virulence.

Pathogen	Clp protease component.	Reference
Salmonella typhimurium	ClpP	Hensel *et al*., 1995; Yamamoto *et al*., 2001; Webb *et al*., 1999
	ClpB	Turner *et al*., 1998
	ClpX	Yamamoto *et al*., 2001
	ClpY/HslU	Valdivia and Falkow, 1997
Leishmania spp.	ClpB	Hubel *et al*., 1997
Pasteurella haemolytica	ClpX-homologue	Highlander *et al*., 1993
Serpulina hyodysenteriae	ClpC	ter Huurne *et al*., 1994
Listeria monocytogenes	ClpC	Rouquette *et al*., 1996
	ClpE	Nair *et al*., 1999
	ClpP	Gaillot *et al*., 2000
Staphylococcus aureus	ClpX	Mei *et al*., 1997; Coulter *et al*., 1998
Yersinia enterolitica	ClpP	Pederson *et al*., 1997
Streptococcus pneumoniae	ClpC	Polissi *et al*., 1998

Some of the STM mutants have not been further characterized, which means that the attenuated virulence may be due to a polar effect, rather than the disruption of the Clp component.

From the data presented so far in the literature, the Clp protease appears to be able to affect virulence in two ways. (i) The lack of Clp proteins result in a decrease in degradation following accumulation of non-functional proteins, which eventually can lead to cell death. (ii) The Clp proteases also regulate the cellular level of regulators and in several cases the Clp proteins are found to modulate expression of virulence factors.

In *S. typhimurium*, ClpXP regulates the level of RpoS, which is involved in the regulation of several virulence factors and proteins required for survival in the stressful environment inside the host (Webb *et al*., 1999; Fang *et al.*, 1992; Coynault *et al*., 1996). The *lktA* gene of *Pasteurella haemolytica* encodes a species-specific leukotoxin, which kills phagocytic cells (Shewen and Wilkie, 1982). LktA is posttranslationally activated by LktC and the expression of these two genes seems to be inhibited by LapA. The sequence of *lapA* is very similar to that of *clpX* from *E. coli*, indicating that it could be involved in the proteolytic degradation of the positive regulator LktC or the leukotoxin itself (Highlander *et al.*, 1993). In *Listeria monocytogenes*, the ClpC ATPase is important for intracellular survival. Quantitative electron microscopy has shown that a *clpC* mutant is unable to disrupt the phagosomal membrane as rapidly as wildtype cells and the virulence of the mutant is strongly attenuated in mice. Apparently, ClpC is involved in the lysis of the phagosomal membrane and thereby promoting the intracellular survival of *L. monocytogenes* (Rouquette *et al.*, 1996; 1998). Since the ATPases possess chaperone activity, the ClpC could be involved in the refolding of virulence factors or transcriptional regulators of virulence genes during intra-phagosomal stress. Also ClpE from *L. monocytogenes* seems to be required for virulence. Results have shown that in a *clpC* mutant, the transcription of ClpE is strongly stimulated, so ClpC seems to play a role in the *clpE* expression (Nair *et al*., 1999). This kind of regulation between Clp ATPases has not been reported before, but it does suggest a chaperone role for ClpC in controlling CtsR activity. In *Yersinia enterolitica*, ClpP

modulates the transcription of the virulence factor *ail*, possibly by degrading the activator of *ail* (Pederson *et al*., 1997). Since Clp proteins are found in almost all prokaryotes and eukaryotes investigated, it seems likely that more Clp proteins will be found to be involved in virulence. This may also lead to the identification of new virulence factors, which are regulated by the Clp proteases.

Results.

5. Signature-tagged Transposon Mutagenesis

5.1 Mutagenesis of *S. typhimurium*.

The purpose of my work was to identify genes in *Salmonella typhimurium* C5 involved in the stress-response in addition to be required for virulence. In order to identify virulence genes of S*almonella*, I used the Signature-tagged Transposon Mutagenesis (STM) technique (Hensel *et al*., 1995). A suicide vector carrying the mini-Tn5 Km2 with tags were obtained from The Institute for Animal Health, Compton, UK. Attempts were made to transfer these plasmids from *E. coli* S.17.1 λpir to *S. typhimurium* pathogenic strain C5 by conjugation. No exconjugant were obtained after mating periods ranging from 30 min to 24 hours. To test whether this was due to problems with the plasmid or the donor, the plasmid was transferred to *Salmonella gallinarum*, which resulted in exconjugants. Thus, the problem was apparently not due to the plasmid itself, but rather a poor ability of *S. typhimurium* C5 to act as a recipient. Various factors can affect the ability of *S. typhimurium* to act as a good recipient, including the composition of the cell envelope (Watanabe *et al*., 1970; Okada and Watanabe, 1968). The initial basis for the formation of mating aggregates may be the binding of the donor pilus to a receptor on the recipient cell, possibly OmpA, since *ompA* mutants of *S. typhimurium* have been shown to be ineffective as recipients in conjugation (Sanderson *et al*., 1981). However, OmpA is also known to serve as receptor for several phages (Lugtenberg and Van Alphen, 1983) and since I was able to use transduction in later work, it is unlikely that C5 has an *ompA* mutation. Strains with mutations affecting the inner core of the lipopolysaccharide (LPS) are usually poor recipients (Sanderson *et al*., 1981), but the lack of core LPS would also affect the virulence of *S. typhimurium* C5, and as shown later, C5 is virulent in a mouse model. A more likely explanation for the problems using *S. typhimurium* C5 as a recipient could be the host restriction system. Mating experiments with *E. coli* to a *S. typhimurium hsdL* and *hsdSA* mutant, gave approximately 100 times as many exconjugants as did the *S. typhimurium* with the wild-type genes (Sanderson *et al*., 1981). In the original paper describing the STM technique they were able to use *S. typhimurium* NCTC 12023 as recipient, obtaining

1510 exconjugants (Hensel *et al*., 1995), but in a more recent paper, the attempt to use *S. typhimurium* ATCC 14028 only resulted in a transposon library containing 260 signature-tagged mutants (Tsolis *et al.*, 1999). I therefore concluded that the ability of certain *S. typhimurium* strains to take up the plasmid used for STM is impaired and no further attempts to make the *S. typhimurium* C5 STM library was done.

5.2 *Salmonella dublin* STM library

5.2.1 Avirulent SPI-1 and SPI-2 mutants

Since I was unable to construct a *S. typhimurium* C5 STM library, a mutant bank of signature-tagged serotype Dublin 3246 Nal^r mini-Tn5 Km2 transposon mutants constructed by Bispham *et al.* (2001) was used instead. Bispham *et al.* (2001) had infected mice and calves via either the intravenous route or the oral route and identified 4 mutants, which were found to be avirulent in both of the animal models. These 4 mutants were sequenced and the effected genes were found to be highly homologous to previously identified *Salmonella* virulence genes (Table 3; Bispham, personal communication; Bispham *et al*., 2001). Two of the genes (*sseD and ssaT*) are components of the type III secretion system of *Salmonella* pathogenicity island 2 (SPI-2) which is required for systemic infection of this pathogen in mice (Klein and Jones, 2001; Hensel *et al*., 1997; Medina *et al.*, 1999). The SPI-2 genes are induced by growth inside macrophages, and the activation of these virulence genes are obligatory dependent on a multiplicity of environmental cues (Cirillo *et al*., 1998; Bajaj *et al*., 1996). Environmental conditions such as oxygen, osmolarity, pH and Mg^{2+} deprivation in addition to growth state are conditions known to affect the expression of SPI-1 or SPI-2 genes (Bajaj *et al*., 1996; Deiwick *et al*., 1998; Lee and Falkow, 1990; Ernst *et al*., 1990; Deiwick *et al*., 1999).

Table 3. Characterization of tagged genes.

Mutant strain	Gene	Salmonella Pathogenicity Island	Function
29:D11	*sseD*	Encoded at *Salmonella* Pathogenicity Island 2. Component of the type III secretion apparatus of SPI-2.	Secreted protein. Required for intracellular growth in epithelial cells and required for virulence. Localize on the bacterial cell membrane (Bispham *et al*., 2001; Klein & Jones 2001).
26:A12	*ssaT*	Encoded at SPI-2. Component of the type III secretion apparatus of SPI-2.	Required for intracellular growth in epithelial cells and for virulence. Involved in the formation of the membrane-bound subunit of the TTSS (Bispham *et al*., 2001; Hensel *et al*., 1997)
26:C8	*sipD*	Encoded at *Salmonella* Pathogenicity Island 1 (SPI-1).	Secreted protein. Involved in regulation of secretion process and translocation of proteins into target cell. Required for entry into host cell (Kaniga *et al*., 1995; Collazo & Galan 1997).
29:E11	*hilA*	Encoded at SPI-1.	Transcriptional activator of SPI-1 (Bajaj *et al*., 1995). Required for virulence (Penheiter *et al*., 1997)

5.2.2 Growth and gene expression under stress.

The importance of environmental conditions for the expression of the SPI-1 and SPI-2 genes, lead to an investigation of the ability of the four mutants to survive under various conditions of stress. I found that the growth rate of the mutants at 37°C in both rich (LB) and defined media (M9 and M63) was the same as the rate observed for the wild-type. When growth was investigated under different stress conditions; pH (3.5 to 7.5), NaCl (0.1M to 2.5M), H_2O_2 (0.5mM to 15mM) and temperature (45°C or 54°C), the mutants were not impaired in their growth compared to the growth of the wild-type (results not shown). Since growth of the four mutants was unaffected under the conditions of stress tested, and since stress is known to be important for the

induction of SPI-1 and SPI-2 genes, the expression of the four genes, *sseD, hilA, sipD* and *ssaT* was investigated by the use of slot blotting. The wild-type was grown in defined media until mid-exponential phase and then different stress conditions were applied, including low pH, elevated temperature, presence of hydrogen peroxide and high salt concentration. Total RNA was made from samples taken prior to stress and at time intervals after the stress was applied. Slot blots, using riboprobes complementary to mRNA from the four genes, was performed. Hybridization using *sipD* and *ssaT* riboprobes showed equal expression of these genes both when grown without stress and when the cells encountered stress (results not shown). Since s*saU* is the final gene in the *ssaK-U* operon, and the fact that *ssaT* and *ssaU* genes have overlapping reading frames (Hensel *et al*., 1997) a hybridization was also carried out with a riboprobe complementary to this gene. But as observed for *ssaT*, no difference in expression of *ssaU* was seen under the conditions tested. Expression of *hilA* was not detected under any conditions. The quality of the riboprobes was tested by hybridization to chromosomal DNA (results not shown). It has previously been shown that regulation of *hilA* expression by environmental signals is an important method for regulation of the invasive phenotype (Bajaj *et al*., 1995; 1996). It has been demonstrated that the induction of *hilA* expression requires both low oxygen, high osmolarity and slightly alkaline pH (Bajaj *et al*., 1996), which may explain the lack of expression in my experiments. HilA activates the expression of the invasion genes by induction of the regulatory gene *invF* (Bajaj *et al*., 1996; Darwin and Miller, 1999a). The regulation of *hilA* gene expression is also expected to be involved in the regulation of *sipD* expression through InvF. But the slot blots show that under conditions where no *hilA* expression is observed, *sipD* expression is observed, although it is a weak signal (not shown). The *sipD* expression can be explained by recent results, which indicates the presence of a HilA-independent pathway to invasion gene transcription (Rakeman *et al*., 1999). Expression of the SPI-2 gene, *sseD*, was found to be induced 2-3 fold in cells exposed to low pH (Fig. 11).

Previous results have shown that small amounts of SseD are secreted, when the bacteria are grown at pH 5. This protein is thought to be part of a surface structure through which effectors can be translocated (Nikolaus *et al.*, 2001). SPI-2 genes are

induced by growth inside macrophages and the expression of SPI-2 genes encoding structural and secreted proteins was disrupted in macrophages in which vacuolar acidification was blocked (Cirillo *et al*., 1998). Even though Cirillo *et al*. (1998), was unable to show induction of SPI-2 genes by exposure to low pH *in vitro*, the results indicate that low pH induces the expression of the gene.

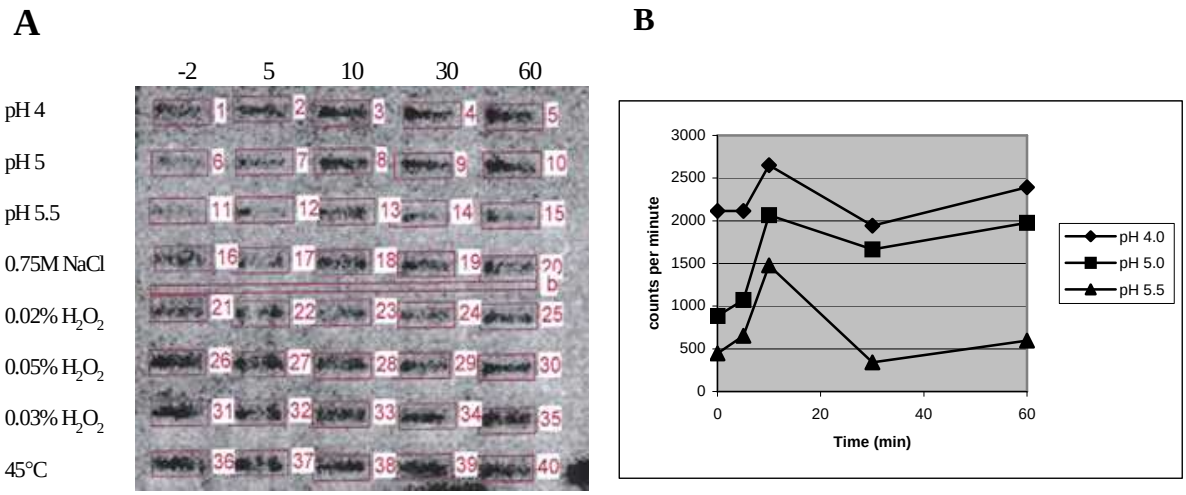


Figure 11. *sseD* expression increases at low pH. Equal amounts of total RNA were blotted onto Zeta-probe nylon membranes. The membranes were prehybridized for 2 hr after which the [^{32}P]-labeled riboprobe was added. A) Slot blot showing *sseD* expression before (-2 min) and after (+5, +10, +30 and +60 min) the wild-type was subjected to the diffrent stress conditions. B) Measurement of Counts Per Minute (CPM) of *sseD* expression when grown at pH4; pH5 and pH5.5 using Packard Instant Imager.

On the basis of Differential Fluorescence Induction (DFI), a SPI-2 gene has been found to be induced more than 400 fold in macrophages (Valdivia and Falkow, 1997). However, the expression of *ssaU*, *ssaT, sipD* and *sseD* appeared very low under the conditions tested in this work. In order to further evaluate the level of *ssaU*, *ssaT*, *sipD* and *sseD* expression, I compared it to the expression of *groEL* and *dnaK* exposed to the same stress conditions. Two dimensional protein gels have previously shown that the two heat shock genes, *groEL* and *dnaK* are highly induced and result in prominent protein spots under conditions of stress and upon infection of macrophages (Morgan *et al*., 1986; Buchmeier and Heffron, 1990). I found that the expression of *groEL* was induced by heat, whereas *dnaK* was induced by heat and

H_2O_2 as already reported (results not shown, Morgan *et al*., 1986; Buchmeier and Heffron, 1990). When compared to expression levels of *groEL* and *dnaK*, I observed an approximately 300 fold lower expression of *ssaU*, *sipD*, *ssaT* and *sseD* (results not shown). The very low level of expression could be due to the very tight regulation of these genes and improper conditions for optimal induction. Due to the few results I obtained with the *sseD* mutant and because other groups were working with SseD function, I decided not to continue my work with this mutant.

5.3 Identification of an avirulent and stress sensitive mutant.

5.3.1 Screening for stress sensitive mutants.

In addition to the four avirulent mutants received from The Institute for Animal Health in Compton, I also obtained the replicate filters, which Bispham *et al*., (2001) had hybridized with the input pool and the recovered pool after injection into mice. When I compared the replicate filters, I found 30 potential avirulent *S. dublin* mutants. In order to identify mutants among these 30 candidates, which were affected in their ability to grow under stress, I tested their growth at 45°C. Only one mutant (LT3975) was found to be impaired in growth compared to the wild-type (Fig. 12).

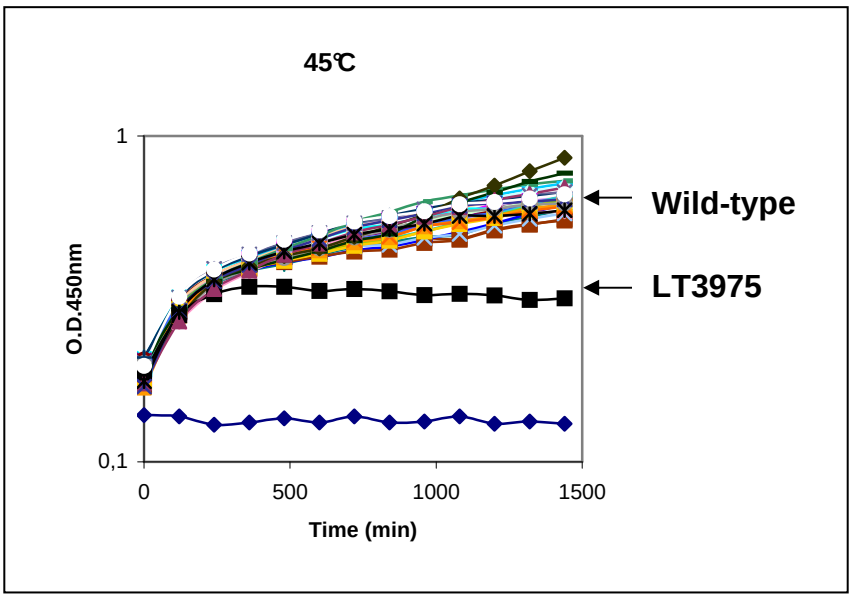


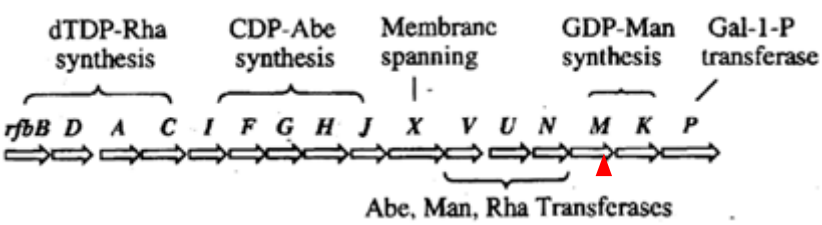
Figure 12. Growth of avirulent *Salmonella dublin* transposon mutants at 45°C. Over night cultures of the 30 strains and wild-type SD3246N were diluted 100 fold in rich media. The growth was followed at 45°C for 24 hr in a Bioscreen (Thermo LabSystems).

I would expect to find more than one gene which is important for both virulence and the ability to survive stress, but several reasons may explain why only one was found: (i) when the bacteria enters its host, it encounter several other different stress conditions which could affect the growth, but only high temperature was used in this procedure. (ii) The screening was probably not sensitive enough, since the growth experiments were carried out in a Bioscreen, which is good for parallel screening of all the strains at once, but unable to dilute the samples. This results in OD_{450} measurements, which peak around 1. The wild-type is able to reach an optical density at 450nm of approximately 3.5, which means that a possible mutant that only grow to an OD_{450} value of 1 will not be found different from the wild-type using the Bioscreen.

5.3.2 Sequencing of LT3975

A Southern blot, using a probe recognizing the transposon sequence, confirmed that it was only inserted once in the LT3975 chromosome (not shown). By use of a primer, which anneals to the transposon in LT3975, the sequence of the disrupted gene was found to have 93% identity to the *rfbM* gene from *S. typhimurium*, a gene that is part of the *Salmonella enterica* O antigen gene cluster (Fig.13A).

A



B

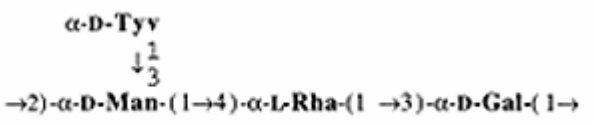


Figure 13. A) *Salmonella typhimurium* O antigen gene cluster. In *Salmonella dublin*, the genes required for abequose synthesis are substituted with genes required for tyvelose synthesis. The insertion of the transposon is marked by a triangle. Figure adapted from Raetz (1996).
B) Structure of the O antigen of *Salmonella dublin*. Abbreviations: Gal, galactose; Man, mannose; Rha, rhamnose; Tyv, tyvelose. Figure adapted from Curd *et al*. (1998).

rfbM encodes the mannose-1-phosphate guanylyl-transferase, which is used for GDP-mannose synthesis. The mannose pathway genes are essentially identical between *S. typhimurium* and *S. dublin* (Xiang *et al*., 1993).

Since the genes in the *rfb* gene cluster probably are arranged in an operon (Jiang *et al*., 1991), it is likely that the insertion of the transposon in the *rfbM* gene have a polar effect on the downstream genes. RfbK is also involved in the GDP-mannose synthesis and the *rfbP* gene product probably plays a role in O antigen export. The N-terminal domain of the RfbP may function as a flippase that deliver the O antigen unit to the periplasmic surface of the inner membrane (Wang and Reeves, 1994). *rfbM* has previously been found by STM to be important for virulence in *Y.enterocolitica* and in *S. typhimurium* (Hensel *et al*., 1995; Darwin and Miller, 1999b). *rfb* mutants are usually found to lack the O antigen and several experiments were performed to investigate whether LT3975 lack the O antigen. Serotyping using Kauffmann-White, revealed that both wild-type and LT3975 could be identified as *Salmonella dublin*. The O antigen constitutes the immunodominant portion of LPS and the structural determinants of O side chains provide the basis for the serological classification of the *Enterobacteriaceae* according to the Kauffmann-White scheme (Kauffmann, 1966). The wild-type and LT3975 did not differ in morphology, even though the lack of O antigen results in rough colony morphology in *Salmonella* (Raetz, 1996). In *S. typhimurium*, the O antigen is necessary for P22 adsorption (Eriksson and Lindberg, 1977), and my work showed that LT3975 was P22 sensitive (results not shown). Taken together, these results indicate that the *rfbM* mutant is capable of synthesizing a wild-type O antigen.

5.3.3 LPS profile.

In order to determine whether the composition of the LPS from the *rfbM* mutant is different from the wild-type, a LPS profile was made (Fig.14). The repeating nature and size-variation of the O side chain can be visualized by separation of LPS on a polyacrylamide gel. Each band in the ladder represents a LPS molecule with one single oligosaccharide unit more or less than the one next to it.

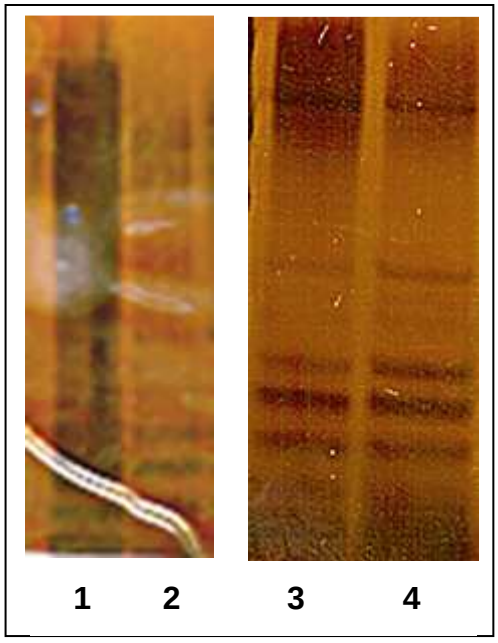


Figure 14. LPS profile of 1) the wild-type SD3246N and 2) the *rfbM* mutant LT3975, 3) 2 fold diluted SD3246N and 4) the *rfbM* mutant LT3975. Samples were subjected to electrophoresis on a 10% SDS-PAGE and the LPS was silver-stained.

The LPS profile reveal that there is no difference in the band pattern between LT3975 and the wild-type (Fig. 14 lane 3 and 4). The same amount of cells was used, but the LT3975 show decreased levels of LPS compared to the wild-type (Fig. 14 lane1 and 2). This result suggest that less LPS is made in the *rfbM* mutant. To confirm this result I investigated agglutination with polyvalent *Salmonella* O anti-rabbit serum, and found that agglutination was observed with both strains, but with the wild-type in a higher dilution compared to the *rfbM* mutant (data not shown). This supports the notion that LT3975 have reduced amounts of LPS compared to wild-type.

5.3.4 The absence of *rfbM* affects growth during stress.

The *rfbM* mutant has already been shown to be impaired in growth at 45°C (Fig.12). This could be due to a slower growth under all conditions, but the growth of LT3975 was not different from that of the wild-type on neither liquid nor solid LB at 37°C (Fig. 15 and not shown). To investigate whether other stress conditions that the bacteria could encounter in the host, affected the growth, the growth of LT3975 was compared to the wild-type at low pH, high salt-concentration and in the presence of hyrdrogen peroxide.

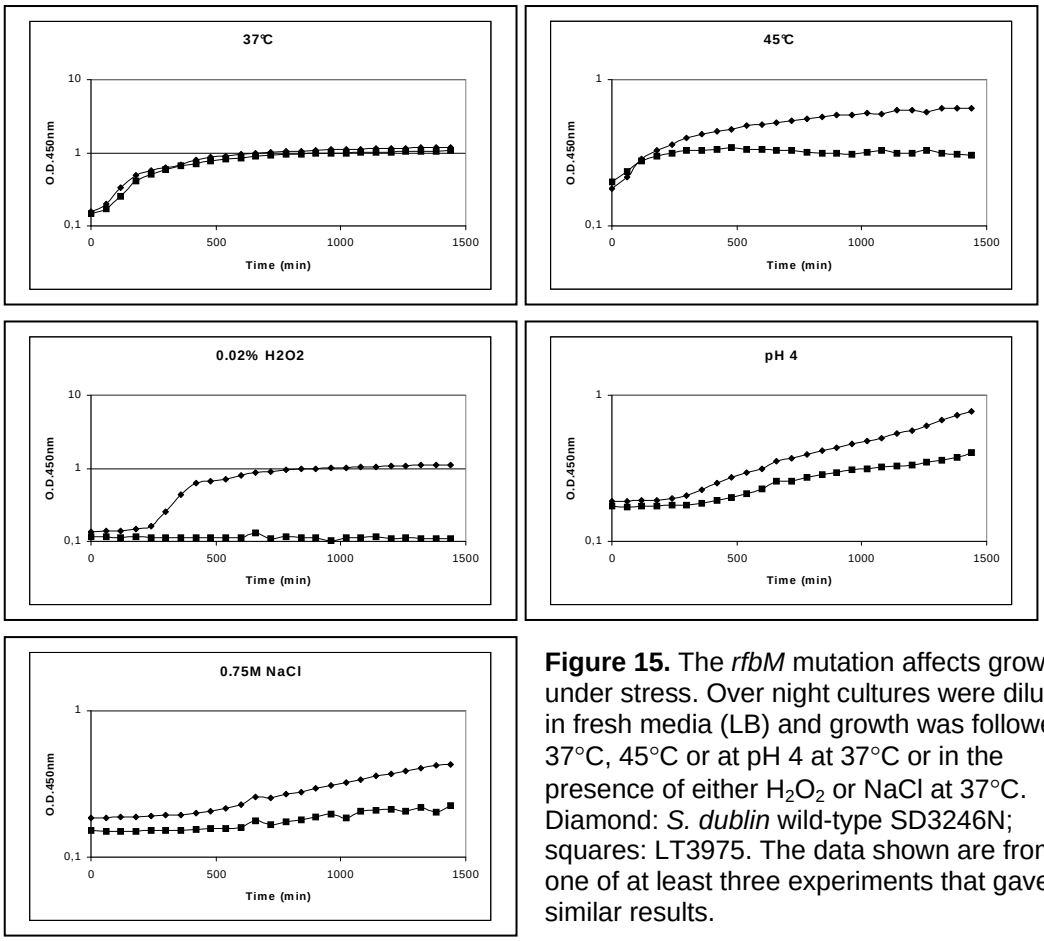


Figure 15. The *rfbM* mutation affects growth under stress. Over night cultures were diluted in fresh media (LB) and growth was followed at 37°C, 45°C or at pH 4 at 37°C or in the presence of either H_2O_2 or NaCl at 37°C. Diamond: *S. dublin* wild-type SD3246N; squares: LT3975. The data shown are from one of at least three experiments that gave similar results.

High temperature and high salt concentration was found to impair the growth of the *rfbM* mutant when grown on plates (not shown). When grown in LB at 45°C and in the presence of 0.02% H_2O_2, it was observed that the mutant was impaired in growth and was unable to reach the same density as the wild-type (Fig.15). The severe effect of H_2O_2 suggested that the *rfbM* mutation in LT3975 might affect the activity of catalase, which catalyzes the conversion of hydrogen peroxide into water and molecular oxygen. However, when I measured the catalase activity, I found that LT3975 was catalase positive (results not shown). Low pH and high salt concentration had a minor effect on the growth of LT3975 (Fig.15).

5.3.5 Virulence of the O antigen mutant

Because LPS is involved in so many aspects of virulence, including resistance to the host complement and phagocytes, essentially all of the genes involved in LPS biosynthesis are essential for virulence, including the ones required for O antigen

synthesis (Galloway and Raetz, 1990; review Raetz, 1996). The results obtained in this work indicate that LT3975 still have the O antigen, but to confirm that LT3975 is actually avirulent, as anticipated from the replicate filters, I infected BALB/c mice orally with $5x10^6$ CFU of the wild-type (SD3246N) or LT3975. The ability of the two strains to colonize the spleen provides a measure of their relative virulence. After 6 days the mice infected with SD3246N died (3 mice) or were sacrificed (5 mice) and high levels ($\geq 10^8$ CFU) of the wild-type were recovered from the spleen. The mice challenged with LT3975 survived 10 days before they were sacrificed (13 mice); in 9 mice no bacteria were recovered from the spleen, whereas in the last 4 mice $2\text{-}3X10^6$ bacteria were recovered. These data clearly demonstrates that the mutation in *rfbM* affects the virulence of *S. dublin* SD3246N. To determine if the lack of virulence is due to differences in sensitivity to macrophage killing, the intracellular survival of these strains was measured within the murine macrophage-like cell line J774A.1 (Fig.16). J774A.1 cells were infected with the two strains at a multiplicity of infection (m.o.i.) of 10. As seen in Figure 16, the lack of RfbM does not seem to affect uptake, survival or replication in this macrophage-like cell line.

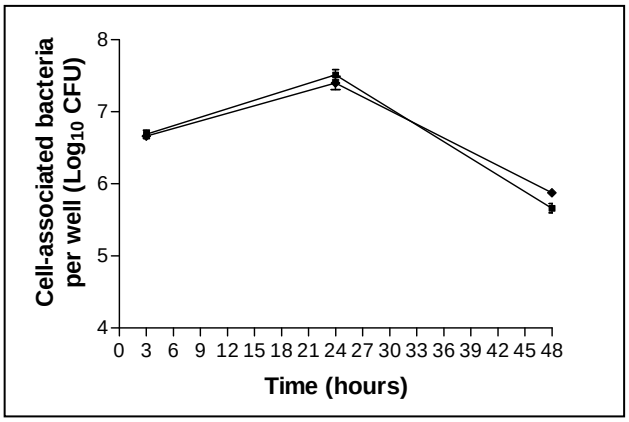


Figure 16. Survival of *S. dublin* SD3246N and LT3975 within J774A.1 macrophages. Macrophages were infected with wild-type 3246N (Diamonds) and LT3975 (squares) at a m.o.i. of 10. The number of viable bacteria at each time point was determined by lysing the macrophages and plating. Results are expressed as the average of five wells plus standard deviation.

Cytotoxicity was determined by measuring the release of the cytoplasmic enzyme lactate dehydrogenase (LDH) from the host cell. Macrophages were infected with the wild-type and LT3975 with an m.o.i. of 10, and *Salmonella*-induced cytotoxicity was examined in macrophages 24 and 48 hours after infection. The results showed that the mutant was as cytotoxic as the wild-type (results not shown).

5.3.6 The oxidative response.

The macrophages are able to convert molecular oxygen into superoxide and other toxic oxygen intermediates, like hydrogen peroxide. This process is known as the oxidative burst and is an important bacteriocidal function of the macrophage. O-specific- and lipid A-segments of LPS are both playing important roles in triggering this oxidative response (Chateau and Caravano, 1997). The activation of oxidative metabolites linked to phagocytosis results in a weak photon emission, i.e. luminescence (Allen and Loose, 1976). This luminescence can be amplified by the addition of a chemiluminiscence substrate as lucigenin. The oxidative burst induced by the wild-type SD3246N and LT3975 was determined by measuring the chemiluminescence response of the macrophage-like cell line J774A.1 cells (Fig. 16). PMA (phorbol myristate, chemical stimulus) and Zymosan A (phagocytosable particular stimulus) stimulate the cells to induce the chemiluminiscence response, and were used as positive controls. As seen in Figure 17, the oxidative response caused by two strains increased to a maximum after approximately 80 min, but the mutant resulted in a slightly reduced oxidative response.

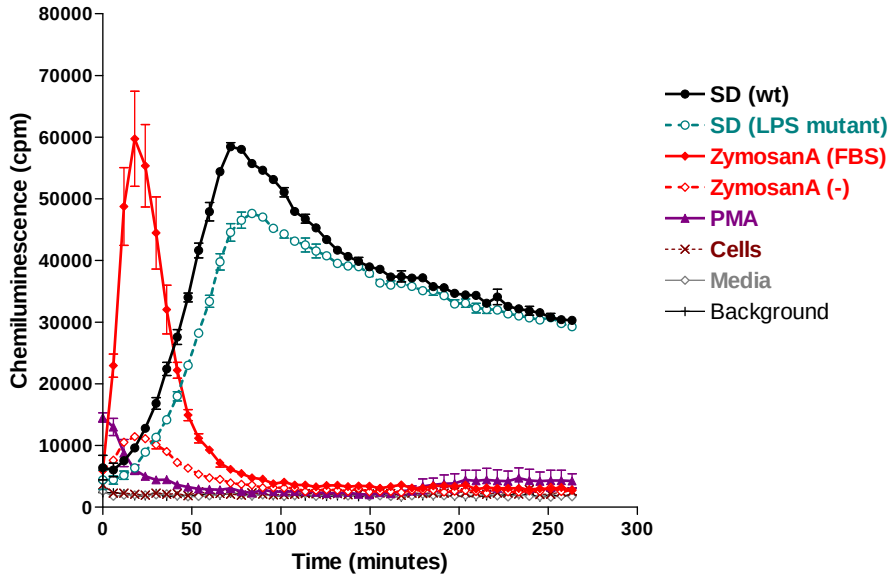


Figure 17. Lucigenin-enhanced oxidative burst chemiluminiscence in J774A.1 murine macrophage cell line with *S.dublin* SD3246N (wt) and LT3975 (LPS mutant). The oxidative burst of Zymosan A (with or without opsonization with Foetal Bovine Serum,FBS) and PMA was used as positive controls. Macrophages (cells) without addition of bacteria and media were used to confirm that the monitored burst was caused by macrophage-bacteria interactions. The data shown are from one of two experiments that gave similar results

6. Identification and characterization of ClpP from *Salmonella typhimurium*.

6.1 Stress- and virulence-related roles of ClpP.

The Signature-tagged Transposon Mutagenesis method was developed to identify bacterial virulence genes and it was originally used on *Salmonella typhimurium* (Hensel *et al*., 1995). One of the genes identified as important for virulence was a homologue of *clpP* from *E. coli*. However, this mutant was not further characterized and it can therefore not be ruled out, that independent second-site mutations or polar effects could affect the phenotype (Hensel *et al*., 1995). ClpP is known from a variety of bacteria to be important for both virulence and growth under various stress conditions (Frees and Ingmer, 1999; Msadek *et al*., 1998; Hensel *et al*., 1995; Gerth *et al*., 1998; Gaillot *et al*., 2000; Kruger *et al.*, 2000). Due to these well-documented stress- and virulence-related roles of ClpP in other bacteria, its function in *S. typhimurium* virulence and stress response was further investigated.

6.2 Cloning and sequencing of the *S. typhimurium* C5 *clpXP* operon.

In *E. coli* the *clpP* gene is the promoter proximal gene in the *clpPX* operon (Gottesman *et al*., 1993). A promoter for *clpX* alone appears to be located in the intercistronic region between *clpP* and *clpX*, but this promoter is relatively weak (Yoo *et al.*, 1994). By a comparison of the *clpXP* operon from *Salmonella typhi* and *E.coli*, I designed degenerated primers, which were used to successfully amplify the *clpPX* operon from *S. typhimurium* C5. The *clpPX* operon was cloned and sequenced. The predicted ClpP and ClpX proteins are 99% and 97,6% identical to the corresponding proteins in *E. coli*, respectively. The functional regions in both proteins are also conserved between *S*. *typhimurium* and *E. coli*. Downstream *clpX* is a putative stem-loop structure, indicating a rho-independent transcriptional terminator (Fig.18).

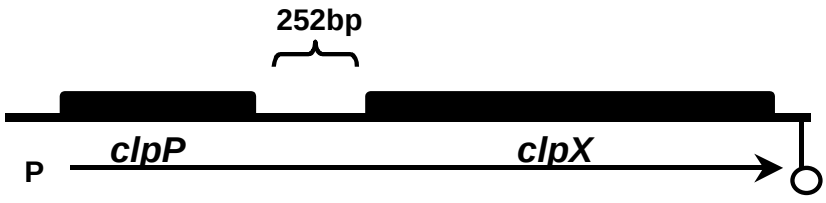


Figure 18. Genomic arrangement of the *S. typhimurium* C5 *clpPX* operon. The horizontal arrow indicates the direction of trancription. The promoter (P) and the potential stem-loop structure (circle) are indicated. The 252 bp intergenic region is shown.

Recently, other groups have also sequenced the operon and deposited the sequence in Genbank (AB033628) (Yamamoto *et al.*, 2001). The sequence I obtained from C5 was similar to the deposited sequence. The *clpXP* intergenic region in *E. coli* is only 125bp, whereas in *S. typhimurium* it is 252bp (Maurizi *et al*., 1990b; Gottesman *et al*., 1993; Yamamoto *et al*., 2001). I found the same putative –10- and Shine-Dalgarno-sequences as found in *E. coli*, which suggests the presence of a weak promoter in front of *clpX*. I found no further promoter elements for *clpX* in the intergenic region present in C5, but absent from *E. coli*. It has been suggested that an 87 bp fragment of *yajO* has been inserted between *clpP* and *clpX* in *S. typhimurium* UK1 (Webb *et al*., 1999), but this does not seem to be the case in *S. typhimurium* C5.

6.3 Construction of the *clpP* mutant LT1100.

To avoid possible polar effect on the *clpX* expression, an in frame *clpP* deletion was made. An 80 amino acid internal fragment was deleted, including the three amino acids which are required for proteolytic activity of ClpP in *E. coli* (Maurizi *et al*., 1990a; Wang *et al*., 1997). This in-frame deletion was generated by allelic exchange, confirmed by sequencing and the resulting *clpP* mutant was named LT1100. To verify that the deletion did not affect the expression of *clpX*, a Western blot, using antibody raised against ClpX, received from Susan Gottesman, was made. The amount of ClpX in wild-type C5 and LT1100 (*clpP*) in both exponential phase (E) and stationary phase (SP) was compared. It was confirmed that the expression of *clpX* was not affected by the deletion in *clpP* (Fig.19).

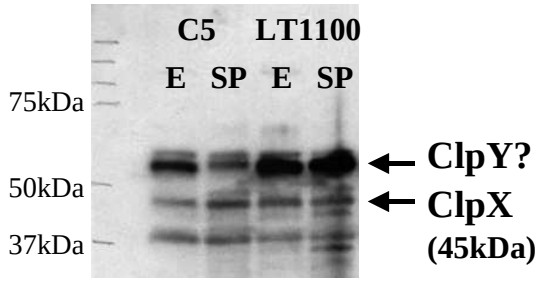


Figure 19. The effect of the *clpP* mutation on ClpX production.
Over night cultures of C5 and LT1100 were diluted 100 fold and grown to mid-exponential phase (E, OD_{600} of 0.4) or late stationary phase (SP, 15 hr growth) in rich media. Equal amounts (5μg) of protein were separated on 11.5% SDS-PAGE and subjected to immunoblot analysis with antibodies raised against ClpX.

A band corresponding to an approximately 53kDa protein was also reacting with the ClpX antibody. The ATP-binding region of ClpY (HslU) exhibit sequence similarity to

that of ClpX and according to information received from S. Gottesman, ClpY would also be seen on a Western blot using this ClpX antibody. ClpY is only 50 kDa (Chuang *et al.*, 1993) and therefore smaller than the protein seen on Figure 19. However, different migration may explain this difference in size. Interestingly, this protein, which possibly is ClpY, is present in increased amounts when ClpP is lacking.

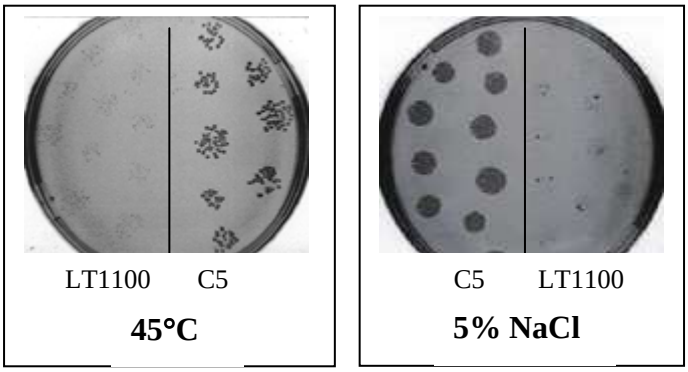
6.4 ClpP is important for the stress-response in *S. typhimurium*.

ClpP is required for stress tolerance in both *Bacillus subtilis*, *Listeria monocytogenes* and *Lactococcus lactis* but in *E. coli*, inactivation of *clpP* does not lead to any obvious phenotype (Frees and Ingmer, 1999; Msadek *et al*., 1998; Gaillot *et al*., 2000; Maurizi *et al*., 1990b). This difference between Gram-positive and Gram-negative bacteria, lead to an investigation of the requirement of ClpP for the *S. typhimurium* stress-response. The growth rate, which influences both stress-response and virulence, was tested for the *clpP* mutant. A growth rate comparable to that of the wild-type was seen in both enriched- (LB)(Fig.20B) and minimal- (M63) broth (results not shown). ClpP synthesis is in *E. coli* induced by heat shock and in *B. subtilis* by heat shock, high osmolarity and oxygen (Volker *et al*., 1994; Kroh and Simon, 1990). I tested the growth of the *clpP* mutant under some of these inducing stress conditions. The growth of LT1100 on plates with a high concentration of salt or high temperature was poor, confirming that ClpP from *S. typhimurium* is also important for survival at these stress conditions (Fig. 20A).

The growth rate of the *clpP* mutant was further investigated under various stress conditions. *Salmonella* are frequently confronted with acid stress, both in the stomach and in the macrophage phagolyzosome (Rathman *et al*., 1996) and it was therefore tested if LT1100 would be affected by a lowering of the pH to 4,5. A decrease in the growth rate of the mutant compared to the wild-type was observed (Fig. 20B). *S. typhimurium* experience osmotic stress in the host (Foster and Spector, 1995) and in agreement with the results observed on plates, liquid media with a high salt concentration (5% NaCl), reduced the growth rate of LT1100 (Fig.20A and B). When the *clpP* mutant and the wild-type strain were subjected to growth at 45°C, the

growth of the mutant was also impaired as already observed on plates (Fig.20A and B).

A



B

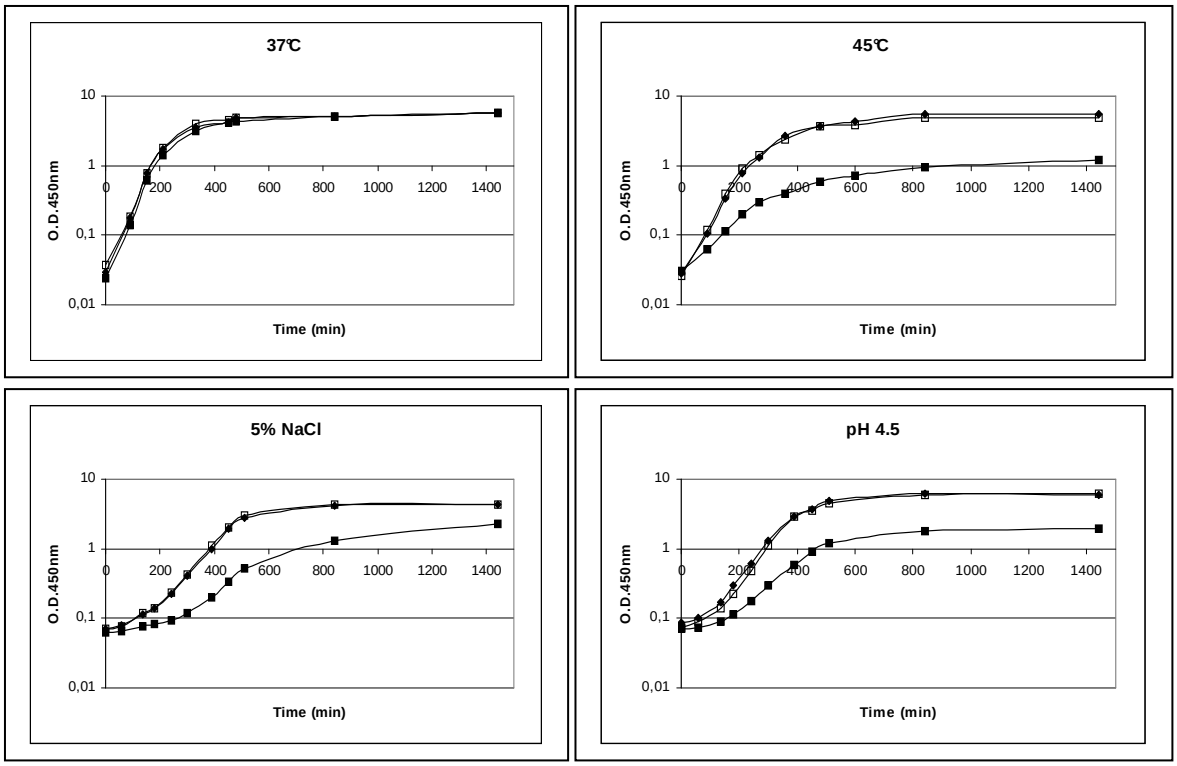


Figure 20. LT1100 exhibit slower growth when exposed to stress. A) Growth of the wild-type C5 and LT1100 (*clpP*) on LB plates at 45°C and 5% NaCl. Over night cultures grown at 37°C were diluted 100 fold and spotted on plates with or without 5% NaCl. The plates were placed at either 45°C or 37°C and allowed to grow over night. B) Growth curves at 37°C, 45°C, 5% NaCl at 37°C and pH 4.5 at 37°C. Over night cultures grown at 37°C were diluted 100 fold in the various media and growth was followed for 24 hr. The data shown are from one of at least three experiments that gave similar results. Diamonds: *S. typhimurium* wild-type C5, Squares: LT1100 (*S.typhimurium clpP*), Open squares: LT1102 (LT1100 *clpP*$^{+}$).

LT1100 was unable to reach the same density during growth as C5, under all conditions of stress tested. These differences in growth were not due to changes in morphology between wild-type and LT1100. The two strains look similar in the microscope under all conditions tested and for LT1100 and C5 the same optical density also corresponds to the same number of colony forming units (CFU) (data not shown). To verify that these differences are due to the lack of *clpP*, the mutation was repaired by cotransduction of a Tn10, which is linked 48% to *clpP*$^+$ into LT1100, resulting in LT1102 (*clpP*$^+$). The growth of LT1102 is the same as that of the wild-type (Fig. 20B) confirming that it is the absence of functional ClpP that results in impaired growth.

6.5 Large colony revertant.

During the work with LT1100, Webb *et al*., (1999) reported that a *S. typhimurium* UK1 *clpP*::Tn10dTc mutation resulted in a small colony morphology (scm$^+$), caused by the overproduction of σ^S (Bearson *et al*., 1996). This small colony morphology that is also observed in a *Y. enterocolitica clpP* mutant (Pederson *et al*., 1997) is not observed with LT1100 cells. From J.W. Foster, I received JF3487, which is the UK1 *clpP*::Tn10dTc (Webb *et al*., 1999). When the *clpP*::Tn10dTc was introduced into the C5 wild-type, the resulting *clpP* mutant had the same scm$^+$ phenotype as JF3487, showing that the difference in colony size is not due to differences in the *S. typhimurium* strains UK1 and C5, but is most likely caused by a secondary mutation, a large colony revertant (lcr), in LT1100. The results obtained with LT1102 show that the secondary mutation does not affect the growth of LT1100 under stress. However, to determine whether the lcr is linked to *clpP* on the chromosome and therefore will be repaired when the *clpP* deletion is repaired, the *clpP*::Tn10dTc was introduced to LT1102, resulting in the strain LT1103 (*clpP*::Tn10dTc). LT1103 has the large colony phenotype, showing that the lcr is still present in LT1102. LT1103 (*clpP*::Tn10dTc) behave like LT1100 (*clpP*) under the various stress conditions tested (data not shown).

To confirm that the scm$^+$ in *S. typhimurium* C5 *clpP*::Tn10dTc is due to the over-production of σ^S, a C5 *rpoS*::Ap mutant was made. This mutant has a large colony morphology, and when the *clpP*::Tn10dTc was placed in this background, the

resulting C5 *clpP*::Tn10, *rpoS*::Ap mutant was still large, supporting the notion that the scm^+ is indeed caused by the high levels of RpoS. LT1100 might carry a promoter-down mutation, which lower the level of expression of *rpoS*. To test whether the secondary mutation affects the amount of RpoS, the level of RpoS was compared in LT1100 and C5 *clpP*::Tn10dTc by the use of RpoS antibody. The result shows that the levels of RpoS in the two *clpP* mutants are the same (results not shown). To investigate whether the activity of RpoS is affected by the secondary mutation, a Tn10, which is linked 68% to *rpoS* was cotransduced into LT1100. If the secondary mutation is in *rpoS*, the scm^+ would be expected in cells receiving the wildtype *rpoS*. Since none of the colonies became small following transduction, it is unlikely that the secondary mutation is in *rpoS* or in its promoter.

6.6 The effect of increased RpoS levels.

6.6.1 Increased RpoS level in the *clpP* mutant.

Degradation of σ^S in *S. typhimurium* and *E. coli* requires the ClpXP protease (Webb *et al*., 1999; Schweder *et al*., 1996; Cunning and Elliott, 1999). Increased levels of RpoS would therefore be expected in LT1100, which was also confirmed in a Western blot, using antibody raised against RpoS. The level of RpoS appears to be increased in the *clpP* mutant, both in exponential phase and also in stationary phase compared to wild-type (Fig. 21). This was most probably because of increased σ^S stability.

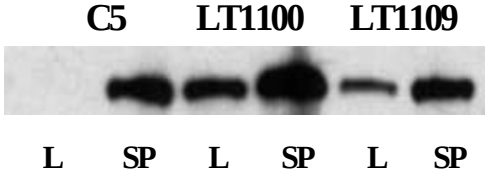


Figure 21. Cellular σ^S levels in wild-type (C5), LT1100 and LT1109. Strains C5 (wild-type), LT1100 (*clpP*) and LT1109 (*mviA*) were grown to mid-exponential phase (L, OD_{600} of 0.4) or late stationary phase (SP, 18hr of growth). Equal amounts (5μg) of protein were separated on 11.5% SDS-PAGE and subjected to immunoblot analysis with antibodies raised against RpoS.

Additionally, a *katE-lacZ* reporter system was used to measure the activity of the *rpoS* gene product. The gene encoding catalase HP-II (*katE*) is controlled by RpoS, and therefore also by factors that modulate the level or activity of this alternative

sigma factor (Tanaka *et al.*, 1997). The fusion was positioned in the C5 (C5K) and LT1100 (LT1100K) chromosome as a single copy by transduction. Increased activity was found in LT1100 (*clpP*) both during logarithmic growth and in stationary phase compared to wild-type cells (Fig.22). LT1102 (*clpP*$^+$) had β-galactosidase levels comparable to the wild-type, suggesting that the secondary mutations does not affect the activity of RpoS. As expected, an *rpoS* mutation (LT1105K) resulted in no β-galactosidase activity (Fig.22).

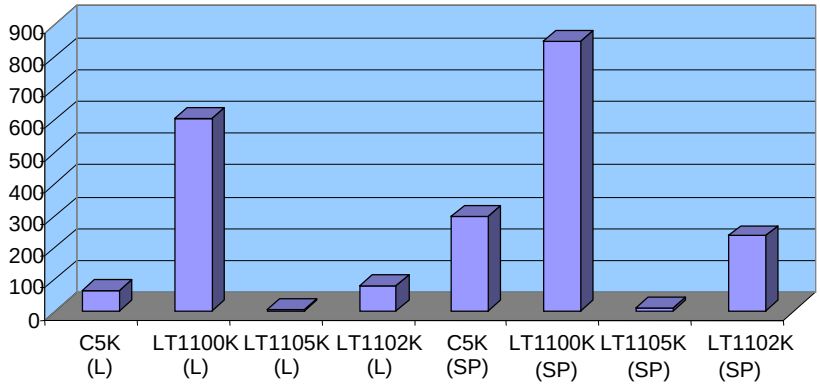


Figure 22. RpoS activity increases in the *clpP* mutant. β-Galactosidase activity measured in wild-type C5K (*katE-lacZ*), LT1100K (*clpP, katE-lacZ*), LT1105K (*rpoS, katE-lacZ*) and LT1102K (*clpP*$^+$, *katE-lacZ*) in exponential phase (L) and late stationary phase (SP). The data presented are from one of three experiments that gave similar results

6.6.2 Increased RpoS level in an *mviA* mutant.

In order to investigate if the increase in RpoS levels is the reason for the impaired growth of LT1100 observed under the stress conditions, an *mviA* mutation was made in C5. Results have shown that MviA, also known as RssB/SprE in *E. coli*, binds RpoS and this binding is required for proteolytic degradation of RpoS by ClpXP (Bearson *et al*., 1996; Muffler *et al*., 1996; Pratt and Silhavy, 1996). The cellular levels of RpoS has been shown to increase in both *rssB* and *mviA* mutants, when compared to the wild-type (Bearson *et al*., 1996; Muffler *et al*., 1996)). I therefore anticipated that an *mviA* mutant would have the same level of RpoS as the *clpP*

mutant, and a comparison of the growth of these two mutants under stress, would clarify whether the impaired growth of LT1100 is due to increased levels of RpoS. A C5 *mviA* mutant gives small colonies (scm$^+$), but large colony revertants (lcr) arise with high frequency, which makes it difficult to perform growth experiments with this strain. An *mviA* mutation was then made in LT1102 (LT1100 *clpP*$^+$), resulting in LT1109, which still possess the secondary mutation. LT1109 maintained the lcr phenotype, which support the observation that the lcr protects the mutants from the effect of increased levels of RpoS. Unfortunately, a Western blot revealed that LT1109 (*mviA*) have increased levels of RpoS compared to wild-type, but not to the same extent as in the *clpP* mutant LT1100 (Fig. 21). This difference suggests that ClpXP is partly able to degrade RpoS without MviA. Although previous results have suggested that RssB is essential for RpoS turnover (Muffler *et al*., 1996), more recent papers conclude that RssB might not be required, but greatly stimulate the degradation (Zhou *et al*., 2001). I could not rule out that the lcr affects this result, but recent results have also shown that the level of RpoS in a UK1 *clpP* mutant is higher compared to a UK1 *mviA* mutant (Webb *et al*., 1999).

6.7 The effect of the *clpP* mutation in the absence of RpoS.

In an attempt to investigate the effect of the *clpP* mutation in the absence of RpoS, the effect of *rpoS* mutations on growth under stress was investigated. An *rpoS* mutation was made in LT1100, giving LT1104 (*rpoS*-*clpP*) and in LT1102, giving the strain LT1108 (*rpoS*). Both strains contain the secondary mutation. The growth of these two mutants, LT1104 and LT1108, was investigated under the same stress conditions as described previously. Both of the *rpoS* mutants were unable to grow in the presence of 5% NaCl (data not shown) as also observed for *E.coli rpoS* mutants (Hengge-Aronis, 2000). The results shown in Figure 23 reveal that while the *rpoS* single mutant (LT1108) behaved as wild-type cells when grown at elevated temperature and low pH, growth of LT1104 (*clpP*, *rpoS*) was impaired under stress, although not to the same extent as the *clpP* single mutant. Thus, my results show that the absence of ClpP affects the growth under stress through both RpoS-dependent and independent mechanisms.

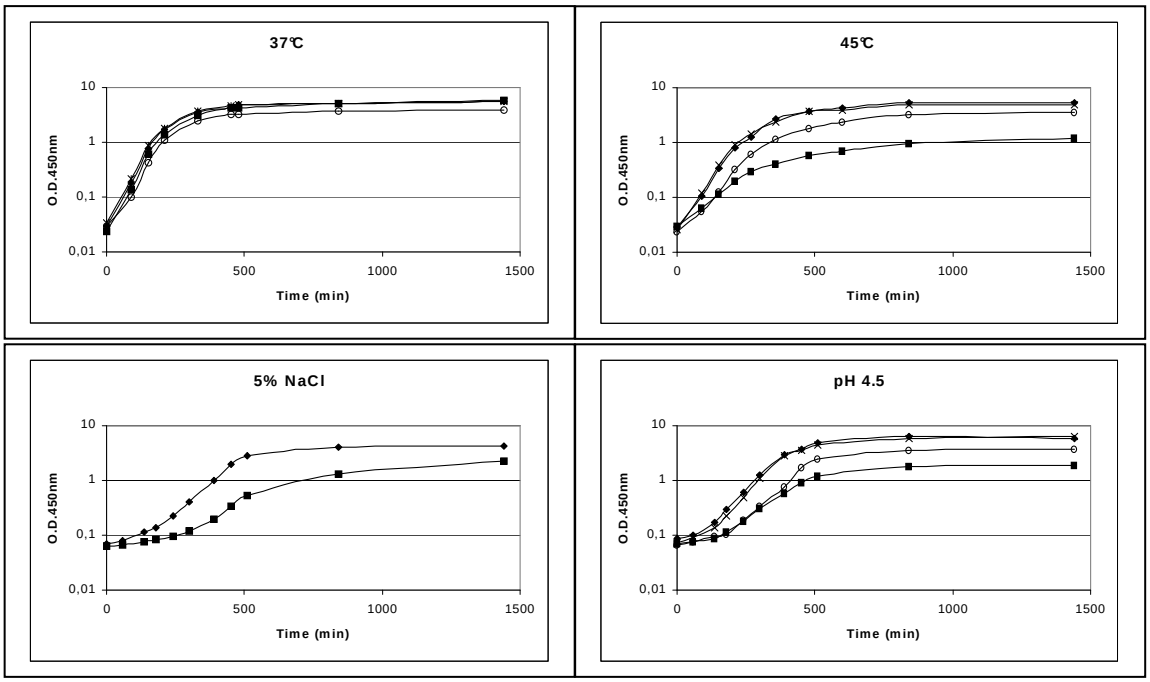


Figure 23. LT1100 exhibit slower growth when exposed to stress. Over night cultures grown in LB were diluted 100 fold in the appropriate media. Growth at 37°C, 45°C, 5% NaCl at 37°C and at pH 4.5 at 37°C was followed for 24hr. Diamonds: *S. typhimurium* wild-type C5, Squares: LT1100 (*clpP*), Open circles: LT1104 (*clpP*; *rpoS*); Crosses: LT1108 (*rpoS*). The data shown are from one of at least three experiments that gave similar results.

6.8 ClpP from *Escherichia coli* is important for growth at low pH.

Although the Clp system in *E. coli* has been studied extensively over the past years, the function of ClpP in the stress response is still relatively unclear, since an *E. coli clpP* mutant show no obvious phenotype under all growth conditions tested (Maurizi *et al*., 1990b). Since the results obtained with LT1100, indicated an important function of ClpP in the stress response of Gram-negative bacteria, the growth of an *E. coli clpP* mutant, AMS6P, was subjected to the same stress conditions as LT1100 (Fig.24).

As it was previously reported (Maurizi *et al*., 1990b), the growth of the *E. coli clpP* mutant was identical to the growth of the wild-type at 37°C. The effect of a shift to 45°C or media containing 5% NaCl was only marginally, but surprisingly, the absence of *clpP* lead to impaired growth when the cells were subjected to low pH (Fig.24). Although the *clpP* mutation in *E. coli* did not affect the growth under stress to the

same level as a *clpP* mutation in *S. typhimurium*, the results suggest that ClpP is important for the ability of Gram-negative bacteria to cope with stress conditions. *E. coli* ClpP might have a particular function when the cells are exposed to low pH.

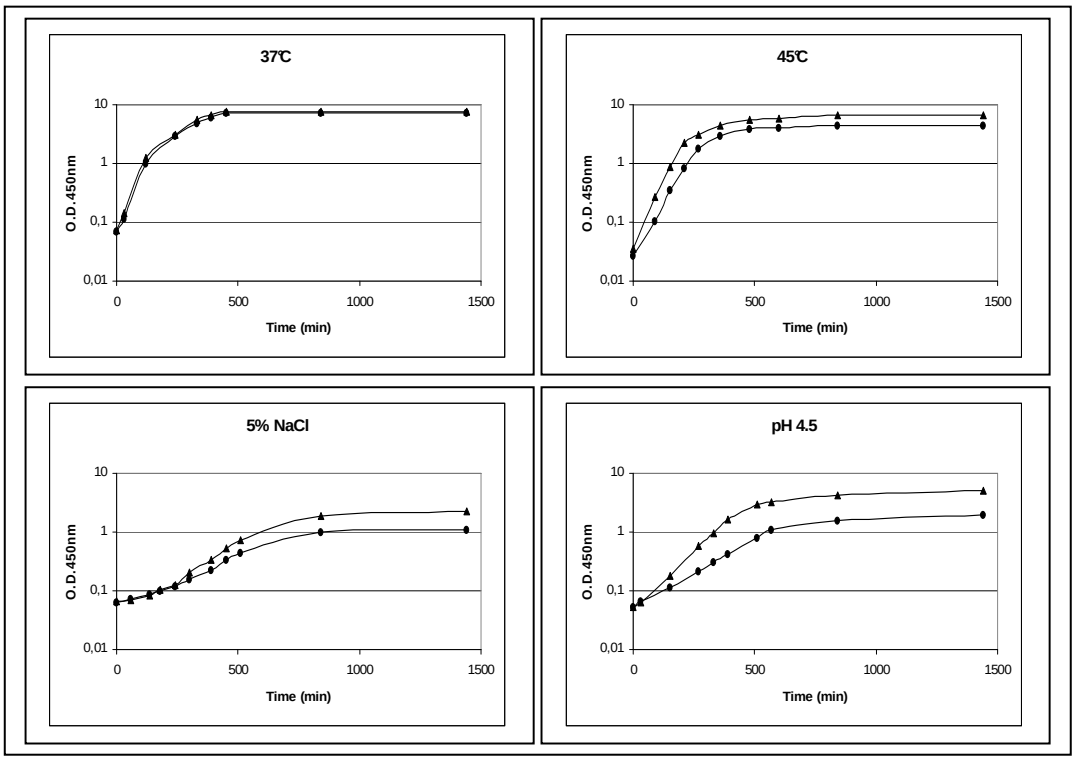


Figure 24. Growth of the *E. coli clpP* mutant is impaired at low pH. Over night cultures of the *E. coli* strains were diluted 100 fold in rich media and grown at 37°C or 45°C or in rich media with either 5% NaCl or pH 4.5 and grown at 37°C. Growth was followed by OD_{450} measurement for 24 hr. Triangles: *E. coli* wild-type AMS6, closed circles: AMS6P (*clpP*). The data shown are from one of three experiments that gave similar results.

6.9. Protein expression in the *S. typhimurium clpP* mutant.

To investigate the proteins expressed in the *Salmonella clpP* mutant when grown under stress, the protein synthesis was analyzed by two-dimensional (2D) gel electrophoresis (Fig.25). The wild-type and LT1100 were grown in defined media at 37°C to exponential phase and then shifted to 45°C, which previously was shown to impair the growth of LT1100 (Fig 20B). Incubation at 45°C continued for an hour

before proteins were labeled by addition of ^{35}S-methionine. Since the absence of ClpP affects the amount of RpoS in the cell (Fig. 21 and Fig. 22), and since it is expected that several proteins are dependent on RpoS. The *rpoS-clpP* double mutant (LT1104) was included in the experiment in order to be able to distinguish the protein pattern caused by the lack of ClpP from that caused by changed levels of RpoS. When the protein patterns of the wild-type strain and LT1100 was compared, enhanced synthesis of 10 proteins and decreased synthesis of 6 proteins was found in the *clpP* mutant (Fig. 25A and B).

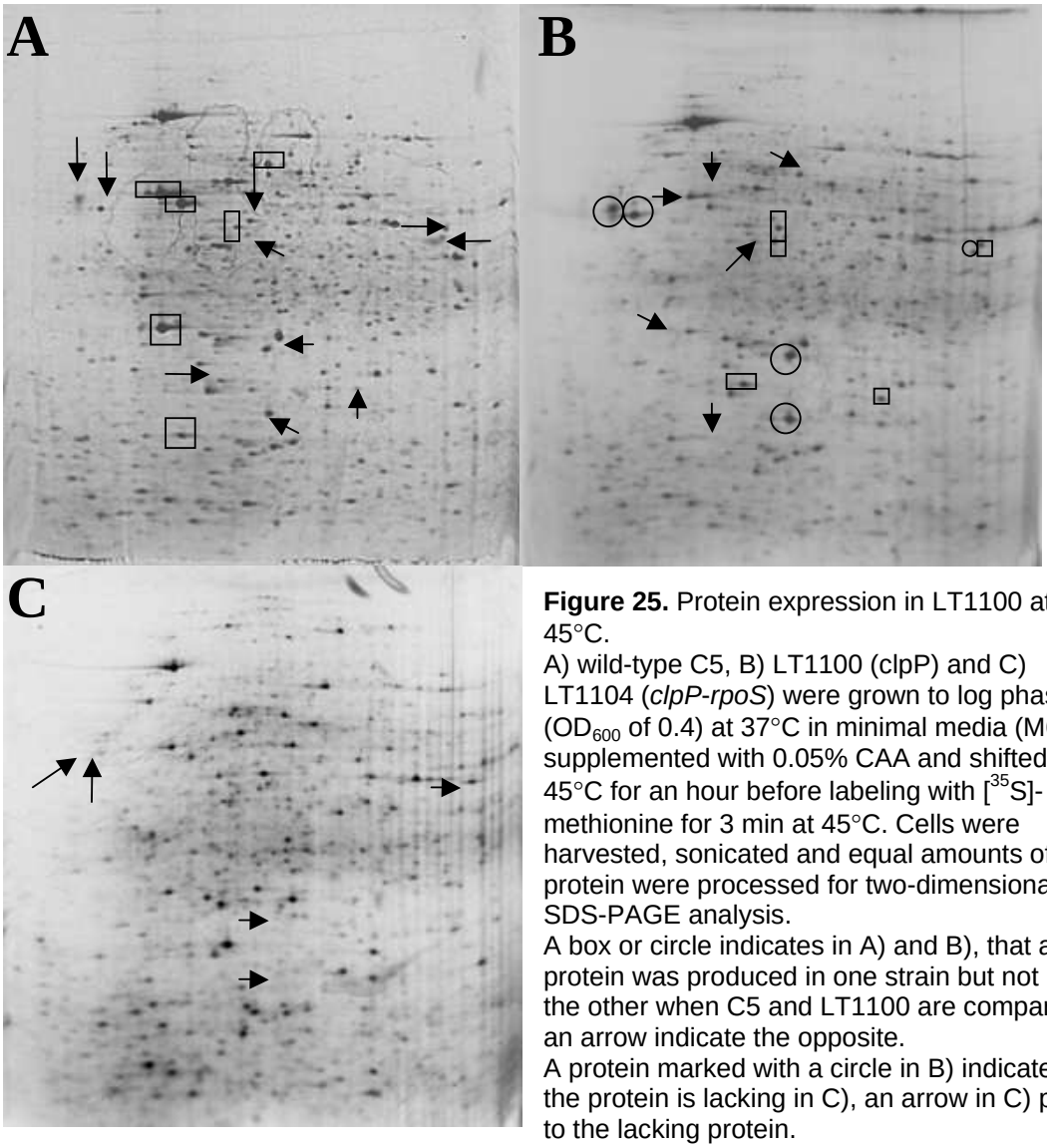


Figure 25. Protein expression in LT1100 at 45°C.
A) wild-type C5, B) LT1100 (clpP) and C) LT1104 (*clpP-rpoS*) were grown to log phase (OD_{600} of 0.4) at 37°C in minimal media (M63) supplemented with 0.05% CAA and shifted to 45°C for an hour before labeling with [^{35}S]-methionine for 3 min at 45°C. Cells were harvested, sonicated and equal amounts of protein were processed for two-dimensional SDS-PAGE analysis.
A box or circle indicates in A) and B), that a protein was produced in one strain but not in the other when C5 and LT1100 are compared, an arrow indicate the opposite.
A protein marked with a circle in B) indicate that the protein is lacking in C), an arrow in C) point to the lacking protein.

Five of these proteins appear to be RpoS dependent, since they are absent from the 2D gel showing the protein pattern from LT1104 (*rpoS-clpP*) (shown as circles on Fig. 25B and Arrows on 25C). The expression of the remaining 11 proteins is affected by the lack of ClpP in an RpoS independent way.

6.10 ClpP degrades misfolded proteins.

B. subtilis, *L. monocytogenes* and *L. lactis clpP* mutants exhibit defects in overall degradation of abnormal proteins, whereas an *E. coli clpP* mutant is essentially unaffected (Kruger *et al*., 2000; Maurizi *et al*., 1990b; Gaillot *et al*., 2000; Frees and Ingmer, 1999; Maurizi1990b). To investigate the turn-over of misfolded protein in LT1100 (*clpP*), both mutant- and wild-type-cells were grown in the presence of the tRNA analogue puromycin, which competes with charged t-RNAs for the A site of the ribosome resulting in premature termination of protein synthesis. The growth rate of LT1100 was impaired compared to the wild-type when grown on plates containing puromycin (Fig.26), suggesting that the *clpP* mutant is more sensitive to puromycin. This could be a result of a reduced ability of the *clpP* mutant to degrade misfolded proteins.

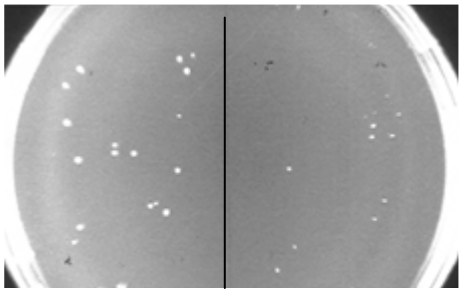


Figure 26. LT1100 is impaired in growth in the presence of puromycin. Exponentially growing cultures of C5 and LT1100 was spotted (10µl) on LB agar containing 65µg/ml puromycin and allowed to grow over night at 37°C.

To examine whether a mutation in the *clpP* gene also affects cellular proteolysis in *S. typhimurium*, the rate of puromycyl-polypeptide degradation at 37°C was determined in LT1100 and in the wild-type as described by Raina and Georgopoulos (1990). Following puromycin treatment of the two strains for 10 min, the cells were pulse labeled for 1min with [^{35}S]-methionine and chased with excess cold methionine. 5 min samples were analyzed for the presence of trichloroacetic acid-soluble

radioactivity. The result, presented in Figure 27, shows that the *clpP* mutant degrades the puromycyl containing fragments at a reduced rate and to a lower extent than the wild-type, showing that the *S. typhimurium clpP* mutant exhibit reduced cellular proteolysis.

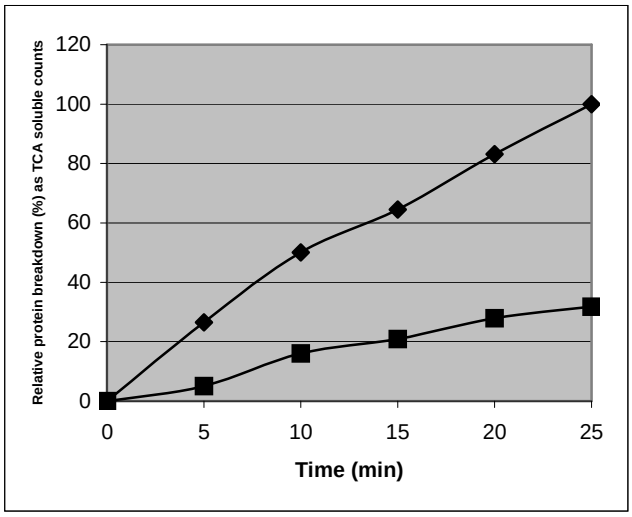


Figure 27. LT1100 exhibit reduced cellular proteolysis. Exponentially growing cultures of LT1100 and C5 cells were incubated with puromycin for 10 min before pulse-labeling with [35S]-methionine. Cells were washed and chased with excess cold methionine. Samples were removed at 5 min intervals following the chase, precipitated with trichloroacetic acid (TCA), and analyzed for TCA-soluble radioactivity by liquid-scintillation counting.
Relative puromycylpolypeptide degradation in the wild-type and the LT1100 is shown.
Diamonds: wild-type C5, squares: LT1100 (*clpP*). The data shown are from one of three experiments, which gave similar results.

6.11 Identification of ClpP regulated genes.

Attempts were made to identify unknown genes in *S. typhimurium*, which are regulated by ClpP. The operon-fusing Mud-*lac* phage is transposon-defective, and requires that the transposase genes are provided in *cis*. When P22 have introduced the MudJ and the transposase genes into the recipient strain, the transposase act on the Mud element, causing its transposition into the recipient chromosome. After transposition the transposase genes are lost, thus, the MudJ is no longer capable of further transposition (Hughes and Roth, 1988). MudJ insertion mutants of *S. typhimurium* LT2 were used for the identification of genes that showed changed expression due to the lack of ClpP. Expression was monitored by measuring the expression of the reporter gene *lacZ*. A P22 lysate from JF3487 (*clpP*::Tn10dTc) was spread onto MaConkey plates containing tetracycline. The MudJ mutants were replica plated onto those plates and onto MaConkey plates without the lysate. After over night incubation at 37°C, the two plates were compared (Fig.28). Two *S. typhimurium* MudJ mutants that changed color due to the *clpP::*Tn10d mutation were

selected, since the expression of the genes in which the MudJ is inserted, probably is regulated by ClpP. Mutant LT1201 (insertion in gene 1) is white on MaConkey, but turns red when a *clpP* mutation is introduced and the LT1202 (insertion in gene 2) is red, but turns white without ClpP (Fig.28).

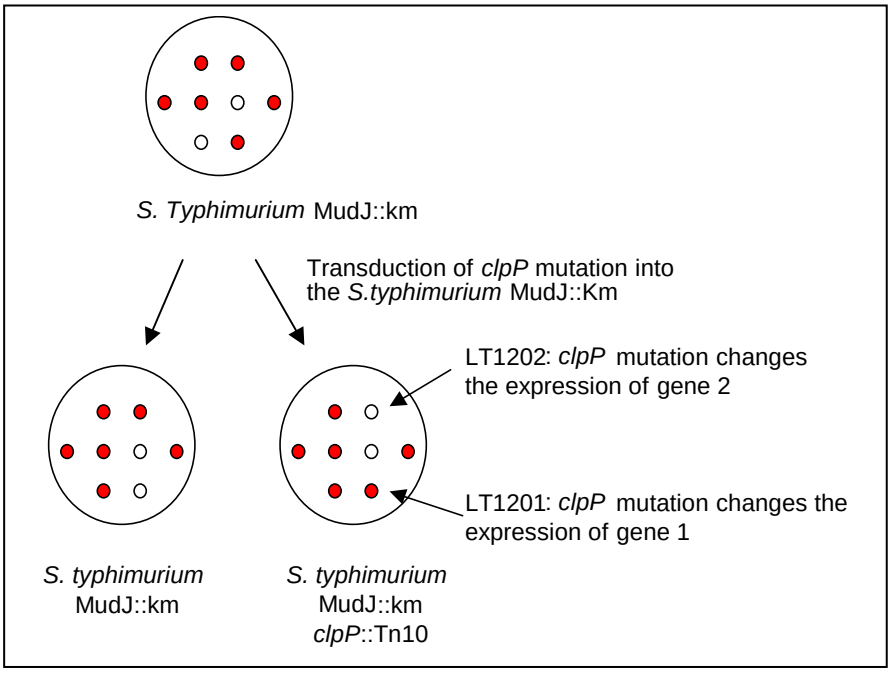


Figure 28. Procedure for identifying genes regulated by ClpP. See text for details.

The two MudJ mutations were transduced to C5 and LT1100 and the β-galactosidase expression was measured (Fig.29). The absence of ClpP clearly affects the expression of the genes in which the MudJ is inserted. The lack of ClpP leads to increased expression of gene 1, whereas it leads to decreased expression of gene 2. To examine whether the lack of ClpP affects the expression of these two genes directly or whether the level of RpoS is involved, an *rpoS* mutation was introduced in the four strains and the expression of β-galactosidase in these mutants are also present in Figure 29. The results show that both gene 1 and 2 are regulated by ClpP through RpoS.

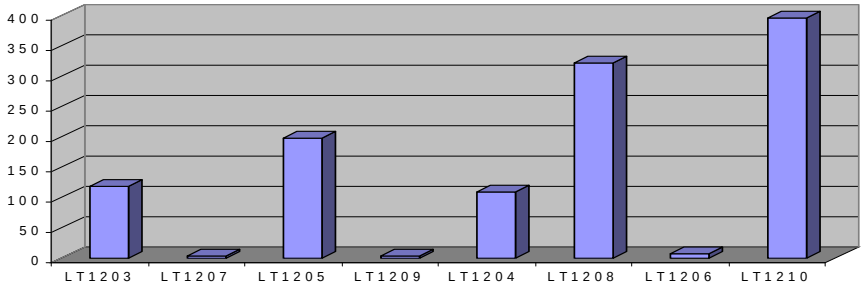


Figure 29. The expression of *iroN* is indirectly regulated by ClpP. Cells were grown to exponential phase (OD_{600} of 0.4) in LB. β-galactosidase expression was measured in:
LT1203 (C5 with insertion in *iroN* (gene 1)); LT1207 (LT1203 *rpoS*::Ap);
LT1205 (LT1100 with insertion in *iroN* (gene 1)); LT1209 (LT1205 *rpoS*::Ap);
LT1204 (C5 with insertion in *iroN* (gene 2)); LT1208 (LT1204 *rpoS*::Ap);
LT1206 (LT1100 with insertion in *iroN* (gene 2)); LT1210 (LT1206 *rpoS*::Ap).

The site of the MudJ insertion in the two mutants was identified and surprisingly, the MudJ was inserted in *iroN* in both mutant LT1201 and LT1202. The MudJ insertions were in the exact same position, but resulted in opposite results. IroN, a gene absent from related species like *E. coli* and *Salmonella bongori*, is an outer membrane siderophores receptor of *S. typhimurium*, belonging to the family of Ton B-dependent outer-membrane receptors (Baumler *et al*., 1998). IroN has a putative Fur-DNA binding site upstream of the *iroN* start codon (Baumler *et al*., 1998). The expression of *iroN* was found to be mediated by ClpP through RpoS, however, RpoS is apparently not involved in the regulation of Fur (Lee *et al.*, 1995), but could be directly involved in the regulation of IroN. Unfortunately, the strain with insertion 2 became unstable, leading to variations in *lac* expression. It seems possible that the stability of insertion 1 is caused by a secondary mutation and this might explain the opposite results when β-galactosidase is measured in the mutants.

6.12 Virulence properties of the *clpP* mutant.

6.12.1 Virulence in mice.

S. typhimurium, which generally causes gastroenteritis in humans, can establish systemic infections in mice which closely resemble typhoid fever in humans (Bakken, 1950). A *S. typhimurium clpP* mutant constructed by transposon mutagenesis had

already been shown to be avirulent in mice (Hensel *et al*., 1995; Webb *et al*., 1999), but to verify that this was due to the lack of ClpP and not caused by a polar effect on *clpX*, the virulence of LT1100 was investigated. To examine the effect of the *clpP* mutation on survival *in vivo*, BALB/c mice were infected orally with $5x10^6$ CFU of the wild-type or the *clpP* mutant. After 5 to 6 days the mice infected with C5 (15 mice) died and high levels ($\geq 10^8$ CFU) of the wild-type were recovered from the spleen. The mice challenged with LT1100 (15 mice) survived 10 days before they were sacrificed and I was unable to detect any *clpP* mutants from the spleens of these mice. These data clearly demonstrate that the *clpP* mutation severely attenuates the virulence, which is consistent with previous results obtained by Hensel *et al*., (1995) and Webb *et al*., (1999). A recent report shows that a *S. typhimurium clpXP* depleted mutant persist in the liver and spleen, unable to cause a systemic infection, after oral challenge with 10^8 cells (Yamamoto *et al*., 2001).

6.12.2 Survival of LT1100 in macrophages

Previous studies have established that *Salmonella* spp. are capable of surviving and replicating within cultured macrophages (Abshire and Neidhardt, 1993; Buchmeier and Heffron, 1989; Carrol *et al.*, 1979). Macrophages are thought to play a critical role in *Salmonella* pathogenesis, both as cells that can provide an environment in which this bacterium can survive and replicate and as cells which, when activated, can mediate host defense responses against the invading bacteria. The ability of *S. typhimurium* to survive and replicate in host cells, including macrophages, is essential for virulence, since mutants that fail to replicate in cultured cell lines are avirulent in animals (Fields *et al*., 1986; Buchmeier and Heffron, 1989; Leung and Finlay, 1991).

As *S. typhimurium* LT1100 is attenuated in virulence in the mouse model, it was investigated whether the mutant was able to survive and replicate inside macrophages. To determine if there was a difference in sensitivity to macrophage killing of the *clpP* mutant LT1100 and the wild-type C5, the intracellular survival of these strains within the murine macrophage-like cell line J774A.1 was measured. J774A.1 cells were infected with C5 and LT1100 at a multiplicity of infection (m.o.i.) of 10. After 3 hours the number of wild-type and *clpP* mutants in the macrophages

was the same, indicating that the uptake was similar for the two strains. The number of intracellular C5 increased 30-fold from 3 to 24 hours. In contrast, the *clpP* mutant grew poorly in macrophages with only a 2-fold increase during the same 21 hours. After 48 hours the number of intracellular C5 had dropped, but this is probably due to the fact that the macrophages were killed by the bacteria, which were then killed by the gentamycin in the media (Fig.30).

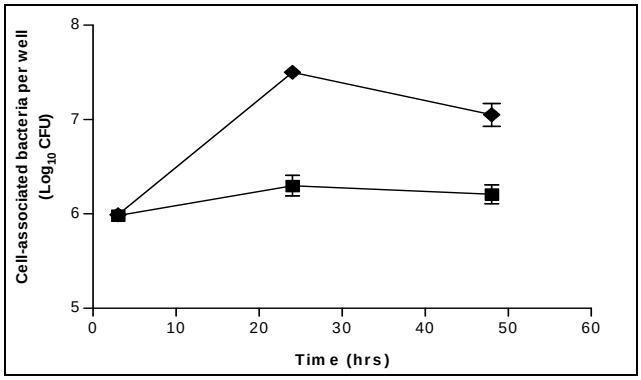


Figure 30. Survival of *S. typhimurium* C5 and LT1100 within J774A.1 macrophages. Macrophages were infected with wild-type C5 (Diamonds) and LT1100 (squares) at a m.o.i. of 10. The number of viable bacteria at each time point was determined by lysing the macrophages and plating. Results are expressed as the average of five wells plus standard deviation.

These experiments indicate that the *clpP* gene is required for optimal growth in macrophages. This is in agreement with recent results, obtained with a *S. typhimurium* χ3306 *clpP* mutant, which in resident peritoneal macrophages show no intracellular growth (Yamamoto *et al*., 2001).

6.12.3 *Salmonella*-induced cytotoxicity.

I investigated whether LT1100 is cytotoxic for the J774A.1 murine macrophage-like cell line by measuring the release of the cytoplasmic enzyme lactate dehydrogenase (LDH) from the host cells. Macrophages were infected with the wild-type and LT1100 with an m.o.i. of 10 and *Salmonella*-induced cytotoxicity was examined in macrophages 24 and 48 hours after infection (Fig.31). 24 hours after infection with C5 there was an 11% reduction in the total number of plate-attached J774A.1 cells. This loss of cells increased to 41% after 48 hours of infection, whereas the loss of *clpP* expression led to a 3-fold decrease of cytotoxicity compared to the wild-type.

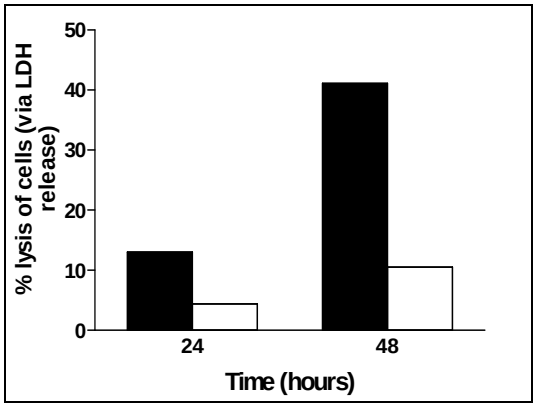


Figure 31. LT1100 show reduces cytotoxicity. Cytotoxicity assessed by lactate dehydrogenase (LDH) release of J774A.1 murine macrophage cell line 24 or 48 hr after infection with *S. typhimurium* C5: black bars; LT1100 (*clpP*): white bars. Cytotoxicity was calculated as described in *Experimental procedures*.

6.13 The oxidative response.

In order to mimic the oxidative killing mechanisms by the oxidative burst in macrophages, the *clpP* mutant and the wild-type were investigated for their sensitivity to H_2O_2 and paraquat (a superoxide anion generator) by disk diffusion assay (Bauer *et al.*, 1966). The results show that the *clpP* mutant is as sensitive as the wild-type to 3% H_2O_2 and 2% paraquat (data not shown). These results correspond to the results obtained by Yamamoto *et al*., (2001) with their *clpP* mutant.

The oxidative burst induced by C5 and LT1100 was determined by measuring the chemiluminescence response of J774A.1 cells. Lucigenin was used to amplify the weak chemiluminescence response caused by activation of oxidative metabolism. PMA and Zymosan A were used as positive controls. *S. typhimurium* C5 was able to induce a chemiluminescence response. The lack of ClpP lead to delayed oxidative response compared to the one observed in the wild-type (Fig.32). The response caused by the wild-type peaks at 72 min after bacteria was added to the macrophages, whereas the response due to the *clpP* mutant peaks 131 minutes later that the wild-type.

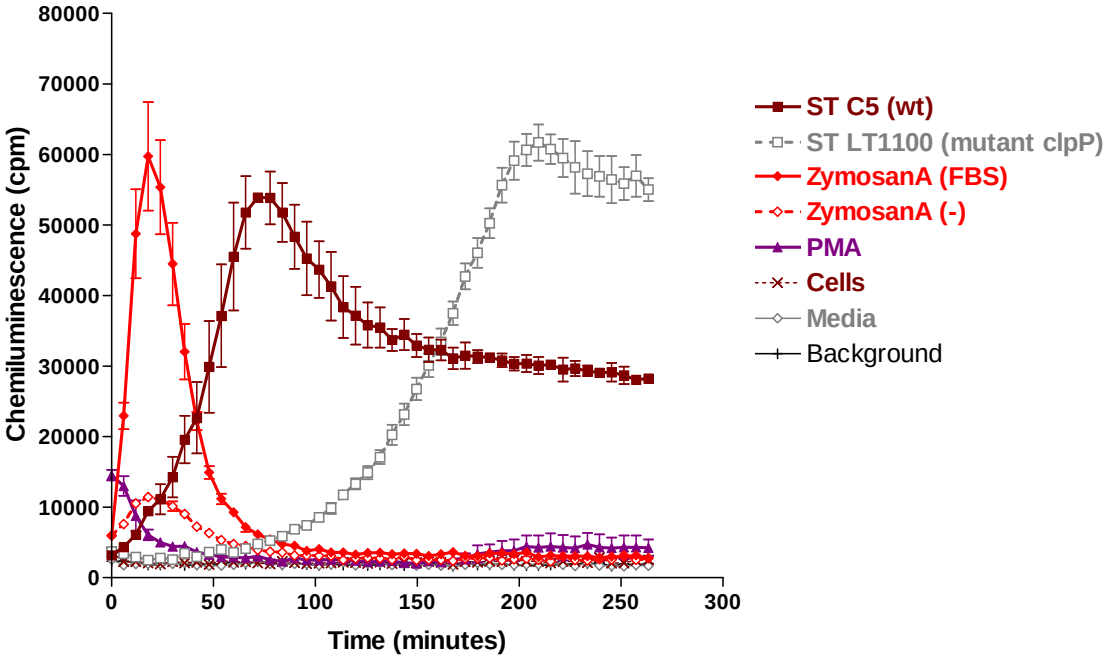


Figure 32. Chemiluminiscence response of the J774A.1 murine macrophage cell line induced by *S. typhimurium* C5 (wt) and LT1100 (*clpP*) (opsonized with Foetal Bovine Serum,FBS) using lucigenin as a probe. The oxidative burst of Zymosan A (with or without opsonization with Foetal Bovine Serum,FBS) and PMA was used as positive controls. Macrophages (cells) without addition of bacteria and media were used to confirm that the monitored burst was caused by macrophage-bacteria interactions. The data shown are from one of two experiments that gave similar results

Discussion.

Salmonella are Gram-negative bacteria that cause gastroenteritis and enteric fever. Their virulence factors require a coordinated expression in order for the bacteria to survive the different host environments, which possess various chemical and physical properties, such as changes in temperature, pH, osmolarity and nutrient availability. The purpose of the work presented here was to investigate genes from *Salmonella*, which are important for the ability of this pathogen to cope with the stress conditions encountered in the host, in addition to being required for virulence. The initial aim was to use the Signature-tagged Transposon Mutagenesis (STM) technique to identify mutants attenuated in virulence. This lead to the identification and characterization of a *S. dublin rfbM* mutant. The use of STM has previously resulted in the identification of a *S. typhimurium clpP* mutant (Hensel *et al*., 1995), but the mutant was not further characterized. In this project a *S. typhimurium clpP* mutant was constructed and the effect of the mutation on virulence and stress response was investigated.

7.1. *Salmonella dublin rfbM* mutant.

The cell wall of Gram-negative bacteria is very different from that of Gram-positive bacteria. The Gram-positive consist of a peptidoglycan layer lying outside the plasma membrane, in contrast, the Gram-negative cell wall is quite complex. They surround themselves with a double membrane and the outermost of these two membranes is asymmetric, with the outer monolayer composed mostly of lipopolysaccharide (LPS) (Lugtenberg and Van Alphen, 1983).

From the Signature-tagged *Salmonella dublin* transposon mutant library constructed by Bispham *et. al.,* (2001), I identified an avirulent, stress sensitive, *rfbM* mutant (LT3975), in which a transposon was inserted after 1202bp of the 1440 bp gene (Appendix 2). *rfbM* encodes the mannose-1-phosphate guanylyltransferase, which is involved in the synthesis of the GDP-mannose, that serves as the activated sugar nucleotide precursor for mannose residues of the O antigen of the lipopolysaccharide (Wang and Reeves, 1994). The genes in the *rfb* gene cluster are thought to be arranged in an operon (Jiang *et al*., 1991), which means that the transposon inserted

in *rfbM* could have a polar effect on the downstream genes, *rfbK,* encoding phosphomannomutase and *rfbP*, encoding galactosyl-1-phosphate transferase. *rfbM* and *rfbK* have overlapping reading frames (Jiang *et al*., 1991), but there is a 1439bp intergenic region between *rfbK* and *rfbP* in *S. typhimurium* LT2, which could contain a promoter, although I found no obvious promoter sequences. The *rfbK* product is also involved in the GDP-mannose synthesis and the *rfbP* product function by initiating synthesis of the O antigen by addition of galactose-phosphate, but it probably also function as a flippase that delivers the O antigen unit to the periplasmic surface of the inner membrane (Wang and Reeves, 1994).

Clinical isolates of *Salmonella* usually possess an intact O antigen and are termed smooth, because of their colony morphology (Schnaitman and Klena, 1993). Loss of O antigen result in a rough phenotype and loss of virulence (Turner *et al*., 1998; Schnaitman and Klena, 1993). Bispham *et al*., (2001) had used the STM technique (Fig.5) on *S. dublin* 3246N and I obtained their replicate filters from virulence experiments in mice. I identified the *S. dublin rfbM* mutant (LT3975) as potential avirulent based on a comparison of these filters and confirmed that the mutant was attenuated in virulence in the mouse model. Due to the attenuated virulence, and the importance of GDP-mannose in synthesizing the O antigen, I anticipated that the mutant was unable to synthesize a proper O antigen, but several results from this work did not confirm that notion. The O antigen is known to be necessary for P22 adsorption, but I found LT3975 to be P22 sensitive, serotyping of the mutant confirmed the presence of both the O12 and O9 antigen, which *S. dublin* usually contain and the mutant does not have the rough phenotype, usually associated with mutations in the *rfb* operon (Eriksson and Lindberg, 1977; Raetz, 1996). In addition, from the LPS profile it was found that the wild-type and the *rfbM* mutant have the same LPS structure. Even though the LPS structure of the two strains was the same, the LPS profile also showed that there was much less LPS from LT3975 compared to the wild-type. This is consistent with my observation that a higher concentration of the *rfbM* mutant than wild-type is required in order to observe agglutination with polyvalent *Salmonella* O anti-rabbit serum. Taken together, these results suggest that LT3975 is capable of synthesizing the same O antigen as the wild-type, but apparently not to equal amounts. A possible explanation is that the cell can use the

GDP-mannose synthesizing genes from the capsular polysaccharide (M antigen) biosynthesis (*cps*) gene cluster. The *cps* cluster in *S. typhimurium* contains structural genes, *cpsB* and *cpsG*, with enzyme functions identical to *rfbM* and *rfbK* (Jayaratne *et al.*, 1994), but the amino acid identity between CpsB and RfbM, and CpsG and RfbK is only 57% and 19%, respectively (Stevenson *et al.*, 1991). Apparently, CpsG and CpsB form part of a pathway leading to the formation of GDP-fucose, via GDP-mannose (Stevenson *et al*., 1991). That *cpsB* and *cpsG* could be able to participate in the synthesis of mannose for the O antigen is supported by the observation that the *rfb* gene cluster is able to restore biosynthesis of GDP-mannose for both O antigen and for capsular polysaccharide in an *E. coli cps* mutant (Jayaratne *et al*., 1994). A decrease in LPS on LT3975 can be explained by either a less efficient mannose synthesis carried out by the *cps* genes or by a polar effect on *rfbK* and *rfbP*, which thereby becomes less effective in the initiation of O antigen synthesis and export of the O antigen. A possible way to determine the effect of the mutation on *rfbK* and in particular *rfbP*, would be to investigate the expression of the genes by analyzing transcription in a Northern blot and the protein amounts in a Western blot. A decrease of LPS can explain the avirulent nature of LT3975. In wild-type *Salmonella*, the C3 component of the host complement deposits on the long LPS O antigen side chain and the terminal components of the complement system is formed too far from the membrane to be able to disrupt it (Joiner *et al*., 1982; Taylor, 1995). However, a decrease in the LPS amount on the cell membrane could lead to a better accessibility, which could allow the complement to be formed closer to the membrane and thereby increase the ability to destroy it and this could explain the avirulent phenotype of LT3975.

Macrophages exert an important role in the host response, including phagocytosis and killing of pathogens. As a response to the interaction with *Salmonella*, the macrophages have the ability to convert molecular oxygen into toxic intermediates, including hydrogen peroxide, a process known as the respiratory burst. LPS plays an important role in triggering this oxidative response of the macrophages (Chateau and Caravano, 1997), and less LPS could therefore explain the decrease in the oxidative response I observed when comparing the LT3975 mutant to the wild-type. LT3975 grew poorly when exposed to H_2O_2, but no effect of the mutation was observed on

uptake and survival in the macrophage. The explanation for this apparent contradiction might be the decreased oxidative response that LT3975 induces, is too low to affect the growth of LT3975.

When I compared growth of LT3975 to wild-type, I found that LT3975 was impaired in growth under various stress conditions. Since LPS is a major constituent of the cell membrane, in *E. coli*, approximately 75% of the outer leaflet is composed of LPS (Rietschel *et al.*, 1994), a decrease in LPS most likely affects the composition of the membrane. Thus, it seems reasonable that this change in membrane composition could explain the mutants increased sensitivity towards stress, in particular high temperature. Recent results show that a H*elicobacter pylori* O antigen mutant is more sensitive to low pH (McGowan *et al.*, 1998). Their explanation for this acid-sensitive phenotype is that LPS may provide a barrier that reduces the proton influx and thereby maintain the proper intracellular pH.

7.2 *Salmonella typhimurium* ClpP.

During its infection cycle*, S. typhimurium* is exposed to the hostile conditions in the environment and in the host, including starvation, low pH, oxygen stress and elevated temperature (Foster and Spector, 1995). During conditions of stress, the cell accumulates abnormal proteins. The Clp proteases are able to remove these damaged proteins, but the protease also plays an important role in the degradation of short-lived regulatory proteins (Goff and Goldberg, 1987; Gottesman *et al*., 1993; Schweder *et al*., 1996; Wickner *et al*., 1994). This proteolytic degradation is required for the cells ability to cope with the very different environments they encounter and components of the Clp protease are found to be important for many pathogens ability to cause disease (Table 2). Recently, a transposon insertion in *clpP* was found to result in attenuated virulence of *S. typhimurium*, but the mutant was not further investigated (Hensel *et al*., 1995). Previous results have described the importance of ClpP for the stress response in Gram-positive bacteria (Frees and Ingmer, 1999; Gaillot *et al*., 2000; Msadek *et al*., 1998), but in Gram-negative bacteria, the role of ClpP in the stress response is not clear, since an *E. coli clpP* mutant has been reported to be phenotypically identical to wild-type under various growth conditions (Maurizi *et al*., 1990b). In several Gram-positive bacteria, ClpP appears to be

important for the degradation of abnormal proteins (Frees and Ingmer, 1999; Gaillot *et al*., 2000; Kruger *et al*., 2000). In contrast, an *E. coli clpP* mutant shows relatively minor defects in the degradation of abnormal proteins (Maurizi *et al*., 1990b). These apparent difference between Gram-positive and Gram-negative bacteria, prompted me to investigate the effect of a *clpP* mutation in *S. typhimurium*.

The results I obtained in this work show that ClpP from *S. typhimurium* C5 is required for the cells ability to cope with different stress conditions, including high temperature, high salt concentrations and low pH. ClpP was also found to play a role in the survival of *E. coli* during stress, but it seems to be limited to growth at low pH. Since stress lead to an accumulation of misfolded proteins in the cell, I tested the importance of ClpP in degrading pyromycyl-containing polypeptides. Since addition of puromycin leads to premature termination of protein synthesis, it is likely to result in an accumulation of misfolded protein. Turnover rates for truncated puromycyl peptides were significantly decreased in the *S. typhimurium clpP* mutant, confirming that ClpP plays a role in the degradation of misfolded proteins. However, some degradation in the *clpP* mutant was still observed, indicating that other proteases than those containing ClpP are able to degrade misfolded proteins as well.

In LT1100 (*clpP*), the level of RpoS is increased compared to wild-type levels. This is consistent with observations stating that the ClpXP protease is involved in the regulation of RpoS (Schweder *et al*., 1996; Webb *et al*., 1999). Since σ^S triggers the induction of more than 50 different genes, many of which have stress-protective functions, it is possible that this could affect the growth of the *clpP* mutant under stress (Schweder *et al*., 1996). When the growth of an *rpoS* mutant and a *clpP,rpoS* double mutant was compared to that of the wild-type, I found that they grew like wild-type cells at 37°C, whereas an increase in temperature or a downshift in pH resulted in impaired growth of the double mutant, when compared to the single mutant and the wild-type. Based on these results I conclude that in *S. typhimurium*, the absence of ClpP affects the growth under stress through both RpoS –dependent and -independent mechanisms. By Two-dimensional protein gel analysis the expression pattern of the *clpP* mutant was investigated. LT1100 was grown at high temperature, which I had shown affected its growth (Fig.20). The high temperature resulted in both

enhanced and diminished levels of expression of proteins by the *clpP* mutant compared to the wild-type. Since several of these proteins were expected to be a result of the increased levels of *rpoS*, they were compared to a 2D analysis of the *clpP-rpoS* double mutant LT1104 grown under the same conditions. Six of the ClpP-dependent proteins were absent from LT1104, showing that these proteins are dependent on RpoS. Although a high number of the ClpP-dependent proteins were through RpoS regulation, proteins were identified, that were ClpP-dependent but RpoS-independent. The amount of 5 of the proteins was increased in the mutant in an RpoS-independent way, suggesting that they could be specific substrates.

The lack of ClpP has profound effect on growth and virulence of *S. typhimurium*. I found that macrophage uptake of wild-type and the *clpP* mutant was the same, but LT1100 grew poorly in the macrophage cell line. LT1100 showed impaired growth *in vitro* under stress conditions and is therefore more likely to be sensitive to the intracellular environment of the macrophage. LT1100 was avirulent in a mouse model and a factor contributing to the reduced virulence of *S. typhimurium* could be the reduced capacity to survive interactions with the macrophages in the mouse. The ability to survive and replicate in the host is important for virulence (Fields *et al*., 1986; Leung and Finlay, 1991), so the lack of replication of LT1100 in the macrophages probably results in the avirulent phenotype in the mouse model. Furthermore, I found that the oxidative burst of J774A.1 in response to LT1100 was delayed compared to exposure to wild-type *Salmonella*. This could indicate a slower engulfment of the *clpP* mutant compared to the wild-type by the macrophage. An investigation using electron microscopy may reveal whether the *clpP* mutant is taken up or situated differently than the wild-type internally.

The results I obtained indicated that a secondary mutation is present in LT1100. Recently, Webb *et al*., (1999) showed that a *S. typhimurium clpP* have a small colony morphology (scm^+) caused by the overproduction of RpoS (Bearson *et al*., 1996), However, my results indicate that the secondary mutation in LT1100 is not in *rpoS*. The presence of a large colony revertant (lcr) in other genes than RpoS has previously been described. Moreno *et. al*., (1999) identified several RpoS-independent genes that suppress the small colony phenotype of *clpP* and *mviA*

mutants. When I performed a Western blot with ClpX antibody, an increase of another protein was seen with LT1100, but not C5 *clpP*::Tn10dTc (scm^+). I propose that this protein is ClpY, based on the fact that the ATP-binding region of ClpY shows sequence similarity to that of ClpX. In addition, information from S. Gottesman confirmed that the ClpX antibody would be able to react with both ClpX and ClpY. ClpY is the ATPase subunit of the ClpYQ ATP-dependent protease (Rohrwild *et al.*, 1996). No increase in ClpY was observed for C5 *clpP*::Tn10dTc (scm^+), suggesting that the secondary mutation in LT1100 affect the level of ClpY. LT1100 could compensate for the lack of ClpP by increasing the level of another protease or ClpP could be directly or indirectly involved in the regulation of ClpY. There is still some degradation of the pyromycyl-containing polypeptides in LT1100 and the ClpYQ protease is known from *E. coli* to participate in the degradation of these polypeptides (Missiakas *et al.*, 1996). ClpYQ show some overlap in substrate recognition with Lon, and it has been suggested that ClpYQ function as a secondary protease for some Lon substrates (Smith *et al*., 1999;Wu *et al*., 1999). A *lon clpP* double mutant in *Salmonella* grows very poorly, a phenotype which is not seen with *E. coli* (Wang *et al*., 1999; Maurizi *et al*., 1990b), although the phenotype of a *S. typhimurium lon* mutant is similar to that observed in *E. coli* (Downs *et al.*, 1986). These observations indicate that ClpP plays a more important role in *Salmonella* than in *E. coli*. Since it appears that the ClpY level is increased in LT1100, it would be interesting to make a *lon* mutation in LT1100, to see if the presence of the secondary mutation would result in a well growing *S. typhimurium lon clpP* double mutant.

Another group recently investigated the virulence of a *S. typhimurium clpPX* mutant. Their results confirm the importance of ClpP for survival and growth within macrophages. Their mutant is unable to cause systemic infection in BALB/c mice, but they find that the mutation results in a persistent infection (Yamamoto *et al*., 2001). I was unable to recover any *clpP* mutants from the spleen of the mice and this difference in the results, could be caused by the 100 fold higher number of bacteria they used for the oral inoculation. However, Yamamoto *et al*., do not mention if their mutants have the scm^+, so it is possible that they have a secondary mutation, which could affect their results. *rpoS* mutations in *Salmonella* are known to result in attenuated virulence (Swords *et al*., 1997). These strains have been used as live

vaccine and the *rpoS* mutants are able to persist in the spleen for at least 29 days (Coynault *et al*.,1996), suggesting that the *clpP* mutant of Yamamoto *et al*., (2001) might contain a *rpoS* mutation as well.

From the results presented here, it seems that ClpP can exert two different functions in *S*. *typhimurium*: (i) The turnover of virulence factors. The 2D analysis revealed that the level of several proteins was increased in the *clpP* mutant. These proteins could be specific substrates of ClpP degradation and potentially important for virulence. The level of one of the known virulence factors, RpoS, was increased in LT1100. The observed increase in RpoS seems to partly affect the growth under stress, and from the results from the 2D gel electrophoresis, it was clear that several proteins was affected in an RpoS –dependent way. RpoS play an important role in regulating virulence gene expression in response to conditions encountered in the host tissue (Guiney *et al*.,1995) and Bearson *et. al.* (1996) have suggested that inappropriate RpoS levels in *S. typhimurium* results in avirulent strains. An *mviA* mutant, which also cause increased σ^S levels, is also known to be avirulent (Benjamin, Jr. *et al.*, 1991). This further supports the need to be able to regulate RpoS activity as the cell encounter high or low stress within the host. A further investigation of the putative substrates present on the 2D gel could reveal other regulators, which are substrates of the Clp protease. (ii) Protein degradation. Accumulation of puromycyl-polypeptides in the cell, suggests that ClpP have an important role in the degradation of abnormal proteins. When synthesis and aggregation of abnormal proteins outpaces degradation, cells accumulate inclusion bodies, which are toxic for the cell (Gottesman, 1996). The decrease in degradation could be part of the reason, the *clpP* mutant show impaired growth under stress. This would be consistent with the observation that the impaired growth of LT1100 was through a partly RpoS-independent mechanism. It seems very likely that both the increase in RpoS levels and maybe other regulators and the lack of degradation of abnormal proteins affect the virulence of the *S. typhimurium clpP* mutant. Based on the results with *clpP* mutants of *S. typhimurium* and *E. coli*, it appears that, as observed in Gram-positive bacteria, ClpP plays an important role in the stress-response in Gram-negative bacteria as well. Apparently ClpP plays a more important role in *S. typhimurium* than in *E. coli*, the results indicate that the *S. typhimurium clpP* mutant is generally more

sensitive to environmental stress than the *E. coli clpP* mutant and this could be due to a reduced ability to degrade misfolded proteins generated under these conditions. From the data I obtained in this work, it is clear that the lack of ClpP from *S. typhimurium* affects its ability to survive stress, degrade pyromycyl-polypeptides, multiply in macrophages and affect virulence in mice. *Salmonella* encounter very different stress conditions during infection of its host and the ability to deal with both the accumulation of abnormal proteins and to regulate the expression of virulence genes may require a more stringent regulation than the one *E. coli* requires

Materials and Methods.

8.1 Bacterial strains.

Strain	Genotype	Source
***S. typhimurium* strains.**		
JF2690	UK1 *rpoS*::Ap	Lee *et al*., 1995
JF2892	UK *mviA*::km^R	Bearson *et al*., 1996
JF3266	UK1 putPA1303::km^R *katE-lacZ* (op)	J.W.Foster, unpublished data
JF3487	UK1 *clpP*1::Tn10dTc	Webb *et al*., 1999
JF3717	UK1 xba-6014::Tn10dCm (48% linked to *clpP*$^+$)	J.W.Foster, unpublished data
JF4843	LT2:zxx-1115::MudJ [plate139AA1MudJpool]-1	J.W.Foster, unpublished data
JF4844	LT2:zxx-1116::MudJ [plate140BH5MudJpool]-2	J.W.Foster, unpublished data
C5	virulent wild-type	Hormaeche, 1979
C5N	C5 Nal^R	This work
C5K	C5 *katE-lacZ*	JF3266 × C5, this work
LT1100	C5 Δ*clpP*$^-$	This work
LT1100K	LT1100 *katE-lacZ*	JF3266 × LT1100, this work
LT1102	LT1100 with Tn10 linked to *clpP*$^+$ (linkage 48%)	JF3717 × LT1100, this work
LT1102K	LT1102 *katE-lacZ*	JF3266 × LT1102, this work
LT1103	LT1102 *clpP*1::Tn10dTc	JF3487 × LT1102, this work
LT1104	LT1100 *rpoS*::Ap	JF2690 × LT1100, this work
LT1105	C5 *rpoS*::Ap	JF2690 × C5, this work
LT1105K	C5 *rpoS*::Ap, *katE-lacZ*	JF3266 × LT1105, this work
LT1108	LT1102 *rpoS*::Ap	JF2690 × LT1102, this work
LT1109	LT1102 *mviA*::km	JF2892 × LT1102, this work
LT1115	C5 *clpP*1::Tn10dTc	JF3487 × C5, this work
LT1203	C5 *iroN*::MudJ (mut.1)	JF4843 × C5, this work
LT1204	C5 *iroN*::MudJ (mut.2)	JF4844 × C5, this work
LT1205	LT1100 *iroN*::MudJ (mut.1)	JF4843 × LT1100, this work
LT1206	LT1100 *iroN*::MudJ (mut.2)	JF4844 × LT1100, this work
LT1207	C5 *iroN*::MudJ (mut.1) *rpoS*::Ap	JF2690 × LT1203, this work
LT1208	C5 *iroN*::MudJ (mut.2) *rpoS*::Ap	JF2690 × LT1204, this work
LT1209	LT1100 *iroN*::MudJ (mut.1) *rpoS*::Ap	JF2690 × LT1205, this work
LT1210	LT1100 *iroN*::MudJ (mut.2) *rpoS*::Ap	JF2690 × LT1206, this work
KP1274	LT2 *(metA22 metE55 val galE*496 *rpsL*120 *xyl*-404 *H*1-*b nm*Γ *H*2-*e n x hsdL*6 *hsdSA*29 (r-m+))	Enomoto & Stocker, 1974

Strain	Genotype	Source
***S. dublin* strains.**		
SD3246N	virulent wild-type Nal^r	Bispham *et al*., 2001
29:D11	SD3246N *sseD*$^-$	Bispham *et al*., 2001
29:E11	SD3246N *hilA*$^-$	J.Bispham, unpublished data
26:A12	SD3246N *ssaT*$^-$	Bispham *et al*., 2001
26:C8	SD3246N *sipD*$^-$	J.Bispham, unpublished data
LT3975	SD3246N *rfbM*$^-$	This work
***E. coli* strains.**		
DH5α	F$^-$ϕ80d*lacZ*ΔM15Δ(*lacZYA-argF*)U169 *deoR* *recA*1 *endA*1 *hsdR*17($r_K^-m_K^+$) *supE*44 *thi*-1 *gyrA*96	Hanahan, 1985
S17-1 λ*pir*	λ*pir* phage lysogen	Miller & Mekalanos, 1988
AMS6	K-12 (λ$^-$ F$^-$ Δ*lac*)	Schultz *et al*., 1988
AMS6P	AMS6 but *clpP*; Cam^r	Schweder *et al*., 1996

8.2 Media.

Luria-Bertani (LB) Broth and M63 medium (Miller, 1992) were prepared as liquid or solid (1.5% agar) media. Unless otherwise stated the strains were grown in LB. Antibiotics were used at the following concentrations in both *S. typhimurium*, *S. dublin* and *E. coli*: Ampicillin, 50 μg ml^{-1}; chloramphenicol, 30 μg ml^{-1}; Nalidixic acid, 20 μg ml^{-1}; Kanamycin, 50μg ml^{-1} and tetracycline, 20 μg ml^{-1}.

8.3 General Methods.

P22 transductions were performed with P22HT105/int$^-$201 as described by Maloy *et al*. (1996). For plasmid constructions, the *E. coli* strain DH5α strain was used. The *S. typhimurium* strain KP1274 was used for transfer of DNA from *E. coli* to *S. typhimurium*. Electroporation and plasmid transformations were performed as described previously by O´Callaghan and Charbit (1990) and Sambrook *et al.*(1989). Plasmid purification was performed according to the manufacturer's instructions (Qiagen, Inc.). β-galactosidase activity was measured according to Miller (1992).

8.4 Mating Methods.

Transfer of plasmids were done either by spreading cells of the donor and recipient on selective medium or mated on millipore membrane filters. 5ml of MgSO4 (10mM) with 500µl donor and 500µl recipient was mixed and filtrated through a Millipore membrane filter (Bedford, MA), which was incubated on a LB plate for 30 min, 1hr, 2hr, 4hr, 8hr and 24hr and resuspended in 5ml MgSO4 (10mM) and plated onto selective plates (nalidixin and kanamycin). Alternatively 500µl of donor and 500µl of recipient were mixed on LB plates containing the appropriate antibiotics. At time intervals (30; 60; 120; 240; 420min and 24 hours) samples were investigated for transconjugants. Using *S. gallinarum* as recipient, transconjugants were observed after 120min.

8.5 Amplification of the *clpPX* operon.

The primers ClpPX-14 (5´ TCAACGAGCTGATGAACC 3´) and ClpX-deg3 (5´ TGCGAATTCAGGKCRYTYTTCGG 3´) were used to amplify the *clpPX* operon (2148bp), which was sequenced.

8.6 Construction of a *S. typhimurium clpP* deletion mutant.

Using a replacement recombination technique a recombinant strain of *S. typhimurium* C5 carrying an 80 amino acid in-frame deletion (from a.a. 96 to a.a. 175) of *clpP* was constructed. By PCR amplification of chromosomal *S. typhimurium* DNA a 750bp fragment carrying part of the upstream region of *clpP* was obtained using:

ClpP-B1 (5´AGTAGATCTCGTCTGCTTACGAAGATCC 3´) and

ClpP-Ec1 (5´AGAGAATTCCTGTCCCATACAAATGGTGC 3´)

while a 642bp fragment carrying the downstream part of *clpP* was obtained using the primers:

ClpP-Ec2 (5´CTCGAATTCCCTGAAGCGGTAGAATACG 3´) and

ClpX-H1 (5´CCTAAGCTTACGCCATTGCTGGTATCG 3´).

The two fragments were digested with *EcoRI/BglII* and *EcoRI/HindIII*, respectively, and cloned into the *BamHI-HindIII* sites of the thermo-sensitive vector pTSA29 (Phillips, 1999), resulting in the plasmid, pLT11, carrying a 1376bp insert.

S. typhimurium C5 was transformed with pLT11 by electroporation and integration was promoted by incubation at 42°C in the presence of ampicillin. To excise the

plasmid from the chromosome, the integrants were grown overnight at 30°C and plated in the presence of ampicillin. The excised plasmid was cured by incubation of the strain in the absence of antibiotics at 42°C. Forty ampicillin sensitive colonies were analyzed by PCR to identify mutants with an internal deletion. One colony gave a single 1376bp fragment, corresponding to the *clpP* gene with a 240bp deletion; the wildtype strain C5 gave a single 1616bp fragment (data not shown). The correct construction of the resulting *clpP* mutant (LT1100) was verified by sequencing the *clpP* gene.

8.7 Growth experiment.

Growth was followed by diluting cultures (grown over night at 37°C in LB) 100 fold into LB and incubating either at 45°C; or at 37°C with or without 5% NaCl or pH reduced to pH 4.5. The optical density was measured at 450nm (OD_{450}).

In plating experiments over night cultures were diluted 100 fold in LB and allowed to grow until OD_{450}=0.4 at 37°C. 10µl of culture was spotted on plates with or without 5% NaCl. Plates were incubated at either 37°C or 45°C over night.

8.8 Immunoblotting.

Western blot analysis using monoclonal anti-σ^S or anti-ClpX antibodies (obtained from Neoclone, Madison and S. Gottesman) was performed essentially as described by Lee *et al*. (1995). Cells were grown to mid-log growth phase (OD_{600}=0.4) or late stationary phase (15 h growth) in LB. Equal amounts (5µg) of protein was loaded in each sample.

8.9 Two-dimensional protein gel electrophoresis.

Two-dimensional SDS-PAGE analysis was performed as described by Spector *et al*. (1986) with minor modifications. Strains were grown in M63 supplemented with 0.05% casamino acids at 37°C until the optical density at 600nm (OD_{600}) was 0.4. The cultures were then transferred to 45°C and allowed to grow for 1hr. Samples were labeled with ^{35}S-translabel (40µCi/ml) for 3 min. In the first dimension proteins were separated using ReadyStrip™ IPG Strips pH 4-7 (Biorad) and in the second dimension an SDS-11.5% polyacrylamide gel was used.

8.10 Measurement of degradation of puromycyl-containing polypeptides.

The experiment was performed essentially as described previously (Frees and Ingmer, 1999; Raina and Georgopoulos, 1990). *S. typhimurium* wild-type and *clpP* mutant cells were grown at 37°C in M63 until the optical density at 450nm reached 0.4. The cells were subsequently incubated with puromycin (200 μg ml^{-1}, Sigma) for 10 min and then labeled with 30 μCi of [^{35}S]-methionine per ml for 10min. The cells were washed and resuspended in M63 containing 500μg ml^{-1} unlabeled methionine. Samples (300 μl) were collected at 5 min intervals and precipitated with 6% trichloroacetic acid. The radioactivity of the acid-soluble fraction was measured by liquid-scintillation counting.

8.11 Signature-tagged transposon mutagenesis.

A spontaneously occurring nalidixic acid-resistant mutant C5-Nal^r, was used in this work. The *E. coli* strain S17.1 λ*pir* and the mini-Tn5 Km2 signature-tagged transposons were kindly provided by T.S. Wallis (Institute for animal Health, Compton). Attempts to construct a transposon mutant bank was done as described previously (Hensel *et al*., 1995).

8.12 RNA Isolation.

S. dublin wild-type was grown in M63 with 0.5% Casamino acid (Difco) and 2μg/ml Nicotinic acid. Growth at 37°C until OD_{450} = 0.4. The culture was then moved to 45°C, or NaCl was added to a final concentration of 0.75M, pH was lowered to 4, 5 or 5.5 or H_2O_2 was added to a final concentration of 0.02%, 0.03 or 0,05%. Samples (20ml) were taken 2 minutes before stress and 5, 10, 30 and 60 minutes after. RNA was extracted by use of the FastRNAkit-BLUE (BIO101) according to the manufacturer's instructions. All RNA preparations were treated with RNase-free DNAse (Roche), extracted once with phenol and once with phenol-$CHCL_3$ (1:1), and resuspended in water.

8.13 In vitro RNA synthesis.

Gene-specific riboprobes were synthesized *in vitro* by transcription of PCR generated DNA templates. The T7 promoter sequence (TAATACGACTCACTATA) was incorporated in the DNA template via the downstream PCR primers.

Oligonucleotides:

ssaT-1: CCAGCAGAAGATTATGATGC

ssaT2-T7: TAATACGACTCACTATAGTAGAATTGAATATCGTACC

sseD-1: AGCATCAATGATTACTGCG

sseD2-T7: TAATACGACTCACTATAGTTGTTGCAGGTCGGCAATCG

hilA-1: AATTTAGCTCGCTAATCTGC

hilA1-T7: TAATACGACTCACTATAGAATTTAGCTCGCTAATCTGC

ssaU-1: TCTGCACTGCTGTTTCTGG

ssaU2-T7: TAATACGACTCACTATAGTACCTTGATACCTTTTAGG

sipD-1: ATTTCAGGCAGCTATTCGG

sipD2-T7: TAATACGACTCACTATAGTTATTACCAGGTAAGGACG

In vitro transcription was performed at 37°C for 1hour in a transcription mixture containing 2µg of the PCR product, 20U of T7 RNA polymerase (Promega), 1x transcription buffer (Promega), 10µM dithiothreitol, 20U of Rnasin (Promega), 0.5mM (each) ATP, UTP, and GTP, 12µm CTP, 2µl of [α-^{32}P]CTP (10µCi/µl, >400Ci/mmol), and water to 20µl. One unit of Rnase-free Dnase (roche) was added and the mixture was incubated for 15 min at 37°C before it was added directly to the hybridization buffer.

8.14 Slot blotting.

The RNA samples were blotted onto Zeta-probe nylon membranes (Biorad) by use of the Bio-Dot SF slot blot apparatus (Biorad). A control containing pooled PCR product from all four probes was also added to the membrane. After a brief rinse in 2X SSC (1X SSC is 0.15M NaCl + 0.015M sodium citrate) (Sambrook *et al*.,1989) plus 0.1% sodium dodecyl sulfate (SDS) for 1min at room temperature, the membrane was air dryed for 10 min and fixed by microwawe heating for 2 min at 950 W. The membranes were prehybridized for 2 h in a hybridization buffer containing 1mM NaCl, 4mM $Na_2P_4O_7$, 5X Reinhardt's solution (544), 1% SDS, 10% (wt/vol) polyethylene glycol 6000, 50mM Tris-HCl (pH 7.5), 250µg of yeast tRNA per ml, and

50% formamide, after which the ^{32}P-labeled riboprobe was added. After an overnight hybridization at 42°C, the membranes were washed twice at room temperature in 2X SSC for 5 min, twice at 65°C in 0,2X SSC plus 1% SDS for 30 min, and twice at 65°C in 0.1X SSC for 30min. After being washed, the membrane was wrapped in plastic wrap. The amount of radioactivity retained by the riboprobes on the membrane was measured in a Packard Instant Imager.

8.15 Sequencing of LT3975.

LT3975 (Nal^r and Km^r) was originally identified in plate 39 well G5 from the bank of signature-tagged serotype Dublin 3246 Nal^r (Bispham *et al*., 2001). Chromosomal DNA was made and the DNA was cut with EcoRI and the fragments were cloned into LITMUS28 (New England Biolabs). Kanamycin resistant plasmid was sequenced with the oligomer P7, which anneal to the inserted transposon.

P7: 5´ GCACTTGTGTATAAGAGTCAG 3´

8.16 LPS profiling.

LPS was extracted by a proteinase K method modified from that of Hitchcock and Brown (1983). Overnight bacterial cultures were harvested with 1 ml of PBS from petri plates, and centrifuged at 13,800 × *g* for 10 min. The Cells were dissolved in approximately 1ml PBS, and adjusted to the same OD_{450} and incubated for 20 min at 60℃. Centrifuged at 13,800 × *g* for 10 min. An aliquot of 100µl of supernatant was heated to 100℃ for 10 min. Ten microliters of proteinase K solution (5 mg/ml; Sigma) was added, and samples were incubated at 60℃ for 1 h and then subjected to electrophoresis on 10% sodium dodecyl sulfate-polyacrylamide gels at 200 V for 1 h. LPS was silver stained by the method of Tsai and Frasch (1982) with a silver stain kit (Bio-Rad Laboratories, Richmond, Calif.).

8.17 Cell assay.

J774A.1 cells were cultivated in RPMI 1640 (5% FCS) and plated to a density of approx. 5×10^5 cells/well (and allowed to adhere for 4 hr). Bacteria were opsonized in foetal Bovine serum (Gibco BRL) and spun onto the macrophages at a m.o.i. of 10. After 1 hr of infection, gentamicin (100µg/ml) was added to kill extracellular bacteria.

After additional 2hr, 23hr and 47hr., the macrophages were lysed in 1 ml 0.1% Triton-X-100 and the bacteria were dilutionally plated onto LB agar for quantitation of CFU.
At the indicated time points during the infections, culture supernatants were collected for analysis as the experimental release samples. Cytotoxicity was quantified colorimetrically with the CytoTox96 lactate dehydrogenase (LDH)-release kit (Promega). The percentage of cytotoxicity was calculated with the formula: 100 × [(experimental release – spontaneous release)/(total release – spontaneous release)], in which spontaneous release is the amount of LDH activity in the supernatant of uninfected cells and total release is the activity in macrophage lysates.

8.18 Oxidative burst chemiluniscence.

The cheminogenic probe lucigenin (Sigma Chemicals Co.) were dissolved in dimethyl sulphoxide (DMSO; Sigma Chemicals Co.) to stock solutions and diluted in HBSS to final assay concentrations of 150μg/ml. Cells were stimulated with PMA (Sigma Chemicals Co.) at 0.2μg/ml and zymosan A(Sigma Chemicals Co.) at 1mg/ml.
Macrophage cells were washed twice in HBSS. A final concentration of approximately $1x10^6$ macrophages/ml was placed on ice for 1 hr. To each chemiluminescent tupe (Röhren tubes, 5ml; Sarstedt) the following solutions were added: 100μl of bacteria (approximately $1x10^7$ CFU/ml, opsonized with normal, heat-treated sera), 400 μl of HBSS buffer, 500 μl macrophage suspension in HBSS (approximately $1x10^5$ cells/ml) and 50μl lucigenin. For stimulation of cells with PMA or Zymosan A, 100 μl of either stimulant were added instead of the bacteria. The controls included: cells without stimulation, HBSS alone without cells, and empty tubes to monitor background reading in the apparatus. Each sample was run in dublicate. The luminometer (AUTOLUMAT LB 953, Berthold) was set at 37°C with reading intervals of 8 min.

8.19 Animal experiments.

Six- to eight-week-old female BALB/c mice were used for all animal infection experiments. The bacterial inoculum (100μl of 5×10^6) was delivered orally (p.o.). 6 days post infection the mice with the wild-type, *Salmonella typhimurium* C5 or *Salmonella dublin* 3246N died or were sacrificed and the bacteria in the spleen were

dilutionally plated onto LB agar for quantitation of CFU. After 10 days the mice with LT1100 (*clpP*) or LT3975 (*rfbM*) were sacrificed and the number of bacteria in the spleen counted.

8.20 Analysis of ClpP regulated genes.

MudJ insertion mutants of *S. typhimurium* LT2 generated by using phage P22 delivery system (Maloy S.R., 1996) was obtained from John W. Foster, University of South Alabama.

MudJ mutants were replica plated to either a Maconkey plate or a Maconkey + tetracycline plate where P22 lysate from JF3487 (*clpP*::Tn10dTc) was spread. The Maconkey plates are used to measure the β-galactosidase expression, which corresponds to the expression of the gene in which the MudJ has inserted. After over night growth at 37°C, the two plates were compared and two MudJ mutants, that changed color due to the *clpP* mutation, were selected. The two MudJ mutations were transduced to *S. typhimurium* C5 and LT1100. The site of the MudJ insertion in the two mutants was identified by PCR amplification and sequencing of the chromosomal DNA at the insertion junction.

A two-step amplification procedure was used. In the first PCR, the arbitrary primer 5´-GGCCACGCGTCGACTAGTCANNNNNNNNNNACGCCC –3´ and the MudJ specific primer 5´-GCACTACAGGCTTGCAAGCCC –3´ were used. Next, 1µl of this PCR was used as the template in a second PCR amplification employing a second arbitrary primer, 5´-GGCCACGCGTCGACTAGTCA –3´ and another MudJ specific primer, 5´-TCTAATCCCATCAGATCCCG –3´ (Surette *et al*., 1999). The PCR product from the second reaction was purified and sequenced.

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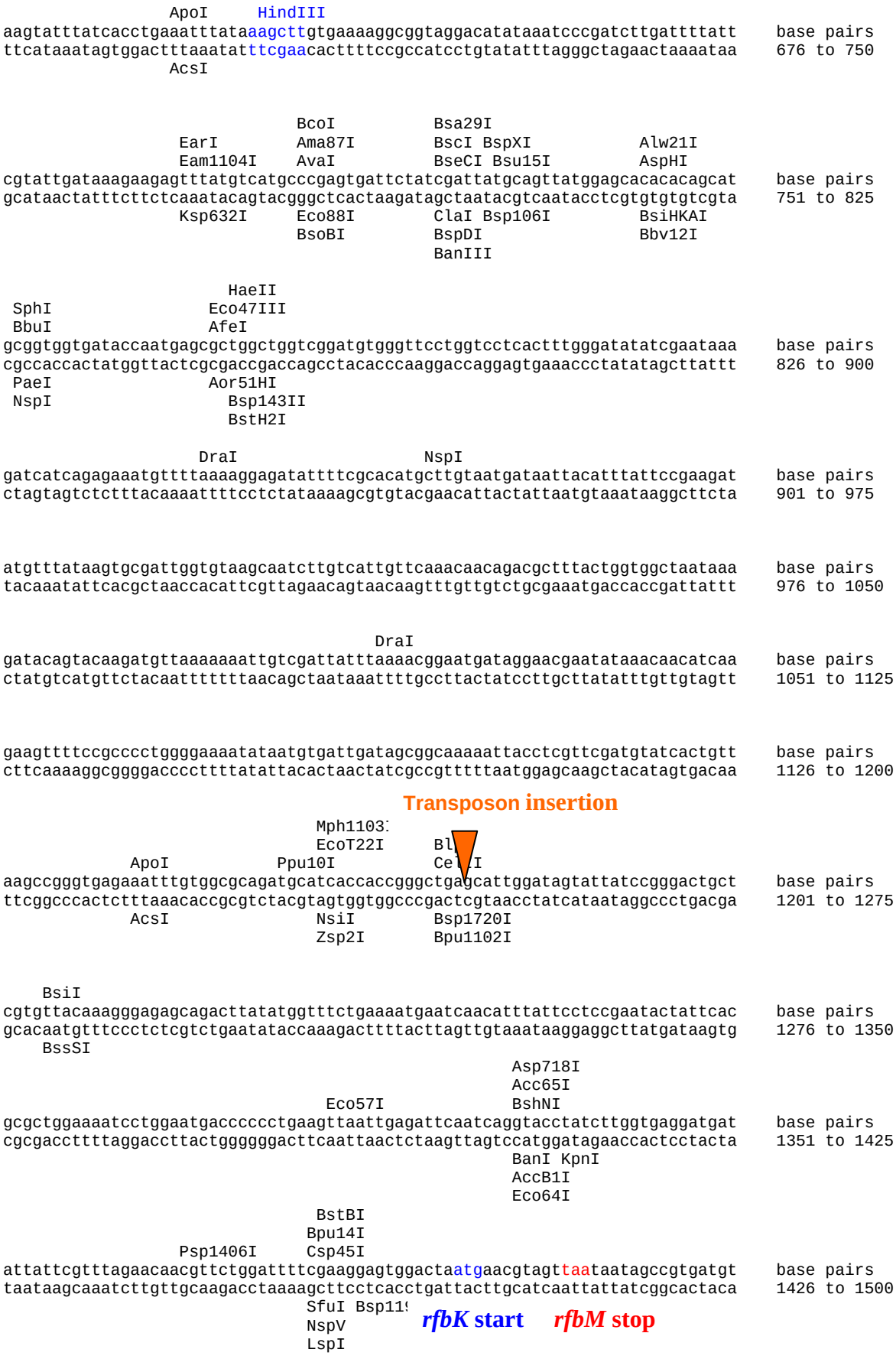
Appendix A.

S.enterica (group B) *rfbM* and *rfbK* (Genbank: X56793).


ApoI
HindIII
base pairs 676 to 750
AcsI
BcoI
Bsa29I
EarI
Ama87I
BscI BspXI
Alw21I
Eam1104I
AvaI
BseCI Bsu15I
AspHI
base pairs 751 to 825
Ksp632I
Eco88I
ClaI Bsp106I
BsiHKAI
BsoBI
BspDI
Bbv12I
BanIII
HaeII
SphI
Eco47III
BbuI
AfeI
base pairs 826 to 900
PaeI
Aor51HI
NspI
Bsp143II
BstH2I
DraI
NspI
base pairs 901 to 975
base pairs 976 to 1050
DraI
base pairs 1051 to 1125
base pairs 1126 to 1200
Transposon insertion
Mph1103I
EcoT22I
ApoI
Ppu10I
AcsI
NsiI
Zsp2I
Bsp1720I
Bpu1102I
base pairs 1201 to 1275
BsiI
base pairs 1276 to 1350
BssSI
Asp718I
Acc65I
Eco57I
BshNI
base pairs 1351 to 1425
BanI KpnI
AccB1I
Eco64I
BstBI
Bpu14I
Psp1406I
Csp45I
base pairs 1426 to 1500
SfuI Bsp119I
NspV
LspI
rfbK start
rfbM stop

Appendix B.

ClpP is involved in the stress response and degradation of misfolded proteins in *Salmonella enterica* serovar Typhimurium.

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Running title: Role of *S. typhimuirum clpP* in stress response

Keywords: *Salmonella enterica* serovar Typhimurium, *clpP*, stress response, misfolded protein

Summary

Components of the ATP-dependent Clp protease complex are found in a wide range of prokaryotic cells and they are often expressed as part of the cellular stress response. To investigate the physiological role of the proteolytic subunit, ClpP in *Salmonella enterica* serovar Typhimurium we constructed an in-frame deletion of the *clpP* gene. Growth experiments reveled that *clpP* is important for the ability of *S. typhimurium* to grow under various stressful conditions, such as low pH, elevated temperature and high salt concentrations. Since the stationary phase sigma factor, RpoS is a target of the Clp proteolytic complex we examined the effect of the *clpP* deletion in the absence of RpoS and observed that growth of the *S. typhimurium clpP* mutant is affected both in a RpoS dependent and independent manner. When we analyzed the degradation of abnormal puromycyl-containing polypeptides we found that ClpP participates in the proteolysis of such proteins in *Salmonella*. These findings prompted us to investigate growth of an *Escherichia coli clpP* mutant and while it was only marginally affected by heat and salt, low pH impaired growth significantly suggesting a particular function of ClpP at this condition. Thus, our results indicate that the *S. typhimurium clpP* mutant is generally more sensitive to environmental stress than the *E. coli clpP* mutant and we propose that this is due to a reduced ability to degrade misfolded proteins generated under these conditions.

Introduction

The continuous requirement for adaptation of bacteria to starvation and physical stress has forced the development of very complex regulatory networks that respond to changes in the environment. During stress, abnormal or misfolded proteins will accumulate in the cell due to denaturation and errors in biosynthesis. The cell responds to this accumulation by increasing the synthesis of both molecular chaperones, which assist the proper folding or refolding of proteins, and of proteases, which degrade the proteins that cannot be refolded (Goff & Goldberg, 1985). Energy-dependent protein degradation is important in both prokaryotic and eukaryotic cells and it is carried out by multimeric protein complexes, such as the proteasome of eukaryotic and archaeal cells (Kessel *et al.*, 1995) and the ATP-dependent proteases of bacterial cells (De Mot *et al.*, 1999). In addition to eliminating abnormal proteins, proteolysis also controls the level of naturally short-lived regulatory proteins

(Mhammedi-Alaoui *et al.*, 1994; Schweder *et al.*, 1996) and therefore is essential for cell homeostasis and optimal metabolic activity (Gottesman, 1996).

In *E. coli*, several ATP-dependent proteases have been characterized (for review (Gottesman, 1996; Miller, 1996; Porankiewicz *et al.*, 1999). Among these is the Clp protease, which together with Lon account for up to 80% of the protein degradation in the cell (Goldberg *et al.*, 1994; Laskowska *et al.*, 1996; Porankiewicz *et al.*, 1999). The Clp protease complex consists of a proteolytic component, ClpP, to which substrate specificity is conferred through association with either of the ATPases, ClpA or ClpX. Beside their functions in proteolysis, both ClpA and ClpX posses chaperone-like activities (Wickner *et al.*, 1994; Wawrzynow *et al.*, 1995). The Clp protease complex mediates the turnover of specific short-lived regulatory proteins (Mhammedi-Alaoui *et al.*, 1994; Schweder *et al.*, 1996), among them the stationary-phase sigma factor, σ^S (RpoS). RpoS regulates the expression of more than 50 genes in the response to stress or the entry into stationary phase (Schweder *et al*., 1996; Hengge-Aronis, 1996; Loewen & Hengge-Aronis, 1994; Hengge-Aronis, 2000). During logarithmic growth the level of RpoS is low, in part due to the degradation by ClpXP (Lange & Hengge-Aronis, 1994). When cells enter stationary phase or encounter various stress conditions, the concentration of RpoS increases as a result of greater resistance to the degradation by ClpXP (Schweder *et al*., 1996; Webb *et al.*, 1999; Zgurskaya *et al*., 1997).

Components of the Clp complex are highly conserved in prokaryotic cells (Maurizi *et al.*, 1990a; Wawrzynow *et al*., 1996). In Gram-positive bacteria ClpP is required for survival under various kinds of stress (Frees & Ingmer, 1999) and it has been shown that ClpP participates in the degradation of misfolded proteins generated under these conditions (Frees & Ingmer, 1999; Kruger *et al.*, 2000; Gaillot *et al.*, 2000). In Gram-negative bacteria the role of ClpP during stress is less clear as indicated by the lack of obvious phenotypes of an *E. coli clpP* mutant (Maurizi *et al.*, 1990b). In recent studies mutants were generated in the *clpP* gene of *Salmonella enterica* serovar Typhimurium (referred to as *Salmonella typhimurium*, Webb *et al.*, 1999; Yamamoto *et al.*, 2001; Hensel *et al.*, 1995). *S. typhimurium* is a facultative intracellular pathogen that upon contact with host cells can promote its own entry (Galan, 1996)

and it was found that *clpP* is required for virulence in a mouse assay (Webb *et al.*, 1999; Yamamoto *et al.*, 2001; Hensel *et al.*, 1995) and for growth and survival within peritoneal macrophages (Yamamoto *et al.*, 2001). Since *S. typhimurium* encounters various hostile conditions during the infection process these findings prompted us to investigate the importance of ClpP for growth in the presence of stress. We find that *clpP* mutant cells have a reduced ability to grow compared to wild type cells when exposed to high temperature, low pH or a high salt concentration. Furthermore, we demonstrate that the *clpP* mutant degrades puromycyl-containing polypeptides to a lesser extent than the wild type indicating that *S. typhimurium* ClpP is important for the degradation of misfolded proteins generated when exposed to stress.

Methods

Media and bacterial strains

The bacterial strains used in this study are shown in Table 1. Luria-Bertani (LB) Broth and M63 medium (Miller, 1992) were prepared as liquid or solid (1.5% agar) media. Unless otherwise stated the strains were grown in LB. Antibiotics were used at the following concentrations in both *S. typhimurium* and *E. coli*: 50 μg ampicillin ml^{-1}; 30 μg chloramphenicol ml^{-1} and 20 μg tetracycline ml^{-1}.

General Methods

P22 transductions were performed with P22HT105/int^{-}201 as described by Maloy *et al*. (1996). For plasmid constructions, the *E. coli* strain DH5α strain was used. The *S. typhimurium* strain KP1274 was used for transfer of DNA from *E. coli* to *S. typhimurium*. Electroporation and plasmid transformations were performed as described previously by O´Callaghan & Charbit (1990) and Sambrook *et al.* (1989). Plasmid purification was performed according to the manufacturer's instructions (Qiagen, Inc.).

Construction of a *S. typhimurium clpP* deletion mutant

Using a replacement recombination technique a recombinant strain of *S. typhimurium* C5 carrying an 80 amino acid in-frame deletion of *clpP* was constructed. By PCR amplification of chromosomal *S. typhimurium* DNA a 750 bp fragment carrying part of the upstream region of *clpP* was obtained using:

ClpP-B1 (5´AGTAGATCTCGTCTGCTTACGAAGATCC 3´) and
ClpP-Ec1 (5´AGAGAATTCCTGTCCCATACAAATGGTGC 3´)
while a 642 bp fragment carrying the downstream part of *clpP* was obtained using the primers:
ClpP-Ec2 (5´CTCGAATTCCCTGAAGCGGTAGAATACG 3´) and
ClpX-H1 (5´CCTAAGCTTACGCCATTGCTGGTATCG 3´).
The two fragments were digested with *Eco*RI/*Bgl*II and *Eco*RI/*Hin*dIII, respectively, and cloned into the *Bam*HI-*Hin*dIII sites of the thermo-sensitive vector pTSA29 (Phillips, 1999), resulting in the plasmid, pLT11, carrying a 1376 bp insert.
S. typhimurium C5 was transformed with pLT11 by electroporation and integration was promoted by incubation at 42 °C in the presence of ampicillin. To excise the plasmid from the chromosome, the integrants were grown overnight at 30 °C and plated in the presence of ampicillin. The excised plasmid was cured by incubation of the strain in the absence of antibiotics at 42 °C. Forty ampicillin sensitive colonies were analyzed by PCR to identify mutants with an internal deletion. One colony gave a single 1376 bp fragment, corresponding to the *clpP* gene with a 240 bp deletion; the wildtype strain C5 gave a single 1616 bp fragment (data not shown). The correct construction of the resulting *clpP* mutant (LT1100) was verified by sequencing the *clpP* gene.

Growth experiment

Growth was followed by diluting cultures (grown over night at 37 °C in LB) 100 fold into LB and incubating either at 45 °C; or at 37 °C with or without 5% NaCl or pH reduced to pH 4.5. The optical density was measured at 450 nm (OD_{450}).
In plating experiments over night cultures were diluted 100 fold in LB and allowed to grow until OD_{450}=0.4 at 37 °C. 10 µl of culture was spotted on plates with or without 5% NaCl. Plates were incubated at either 37 °C or 45 °C over night.

Immunoblotting

Western blot analysis using monoclonal anti-σ^S antibodies (obtained from Neoclone, Madison) was performed essentially as described by Lee *et al*. (1995). Cells were

grown to mid-log growth phase (OD_{600}=0.4) or late stationary phase (15 h growth) in LB. Equal amounts (5 μg) of protein was loaded in each sample.

Two-dimensional protein gel electrophoresis

Two-dimensional SDS-PAGE analysis was performed as described by Spector *et al*. (1986) with minor modifications. Strains were grown in M63 supplemented with 0.05% casamino acids at 37 °C until the optical density at 600 nm (OD_{600}) was 0.4. The cultures were then transferred to 45 °C and allowed to grow for 1 hr. Samples were labeled with ^{35}S-translabel (40 μCi ml^{-1} = 1•48 x 10^6 Bq ml^{-1}) for 3 min. In the first dimension proteins were separated using ReadyStrip™ IPG Strips pH 4-7 (Biorad) and in the second dimension an SDS-11.5% polyacrylamide gel was used. Results presented are representative of two independent experiments.

Measurement of degradation of puromycyl-containing polypeptides

The experiment was performed essentially as described previously (Raina & Georgopoulos, 1990). *S. typhimurium* wild type and *clpP* mutant cells were grown at 37 °C in M63 until the optical density at 450 nm reached 0.4. The cells were subsequently incubated with puromycin (200 μg ml^{-1}, Sigma) for 10 min and then labeled with 30 μCi (1•11 x 10^6 Bq ml^{-1}) of [^{35}S]-methionine per ml for 10 min. The cells were washed and resuspended in M63 containing 500 μg unlabeled methionine ml^{-1}. Samples (300 μl) were collected at 5 min intervals and precipitated with 6% trichloroacetic acid. The radioactivity of the acid-soluble fraction was measured by liquid-scintillation counting.

Results

ClpP is important for growth under stress conditions.

With the aim of investigating the physiological role of ClpP in *S. typhimurium* C5, we constructed a *clpP* mutant (LT1100) by deleting an internal fragment corresponding to 80 amino acid, including the three amino acids known to be required for the proteolytic activity of ClpP in *E. coli* (Maurizi *et al.*, 1990a; Wang *et al*., 1997). By Western-blot analysis using a ClpX antibody we confirmed that the deletion did not affect the expression of *clpX* located downstream of *clpP* (data not shown). When we

investigated the growth of LT1100 at 37 °C the growth rate was comparable to that of the wild type in both enriched (Fig. 1) and minimal broth (data not shown). However, when mutant cells were shifted to 45 °C, the growth was impaired compared to the wild type as observed by a reduction in the growth rate and by the inability to reach the same density as the wild type even after 24 hours (Fig. 1). A high salt concentration (5% NaCl) as well as low pH (pH 4.5) also reduced the growth rate of the *S. typhimurium clpP* mutant (Fig. 1). Furthermore, when mutant and wild type cells were plated either at 45 °C or in the presence of 5% salt the ability of the mutant to form colonies was greatly reduced (Fig. 2). To verify that these differences are due to the lack of *clpP*, we repaired the deletion by transducing the *clpP*$^+$ allele together with Tn10 from JF3717 into LT1100, resulting in LT1102 (*clpP*$^+$). Under all conditions tested LT1102 grew like wild type cells (Fig. 1), confirming that it is the lack of functional ClpP that affects the growth during stress. Thus, our results reveal that the growth of the *S. typhimurium clpP* mutant is impaired when exposed to stress.

In both *S. typhimurium* and *E. coli*, the ClpXP protease is involved in the regulation of the level of RpoS by degradation and a *clpP* mutation results in increased concentrations of RpoS (Schweder *et al*., 1996; Webb *et al*., 1999). By Western blot analysis we confirmed that the level of RpoS is increased in LT1100 compared to the wild type (Fig. 3). Additionally, we followed RpoS activity by measuring the expression of the RpoS regulated gene, *katE*, using a *katE-lacZ* fusion and we found increased activity in the *clpP* mutant both during logarithmic growth and in stationary phase compared to the wild type (results not shown). To investigate whether this increase in RpoS concentration is causing the impaired growth of the *clpP* mutant, we transduced *rpoS*::Ap into LT1100, resulting in the double mutant LT1104 (*clpP*,*rpoS*). To compare the effect due to RpoS we also transduced the *rpoS*::Ap into LT1102 yielding LT1108 (*rpoS*). The growth of the two mutants, LT1104 and LT1108, was examined under the same stress conditions as the *clpP* mutant (Fig. 1). In the presence of a high concentration of salt both of the *rpoS* mutants were unable to grow (data not shown) as has been observed previously in *E. coli* (Hengge-Aronis, 2000). At elevated temperature and low pH the *rpoS* single mutant (LT1108) behaved as wild type cells whereas growth of the *clpP, rpoS* double mutant (LT1104) was impaired, although not to the same degree as the *clpP* single mutant (Fig. 1).

Thus, our results show that in cells exposed to stress the absence of ClpP affects the growth both through RpoS-dependent and independent mechanisms.

During the course of these experiments we observed that LT1100 had a normal colony size. In a recently reported study, a *S. typhimurium clpP* mutant displayed a small colony morphology caused by the overproduction of RpoS (Webb *et al*., 1999) suggesting that LT1100 carries a secondary mutation. We verified this notion by transducing the transposon disrupted *clpP* allele *clpP*::Tn10dTc (Webb *et al*., 1999) into C5, and found that the resulting C5 *clpP* mutant had a small colony morphology whereas tranduction into LT1102, which carries the secondary mutation in addition to the $clpP^+$ allele, still resulted in a large colony morphology (data not shown). Thus, LT1100 carries a secondary mutation resulting in a normal colony size. To test whether this mutation affects the amount of RpoS, we compared the level of RpoS in LT1100 and C5 *clpP*::Tn10dTc using the RpoS antibody and found that the same amount of RpoS was present in the two *clpP* mutants (results not shown). Furthermore, growth of LT1102 was identical to growth of C5 (Fig. 1) confirming that the impaired growth observed with LT1100 is not a consequence of the secondary mutation but rather it is caused by the lack of ClpP.

The absence of ClpP in *E. coli* also affects growth during stress

Although the Clp system in *E. coli* has been studied extensively over the past years only limited information has been published concerning the importance of these proteins for growth during stress (Maurizi *et al*., 1990b). The impaired growth we observed for LT1100 when exposed to stress prompted us to investigate the growth of an *E. coli clpP* mutant using the same experimental conditions. As was previously reported (Maurizi *et al*., 1990b) growth of the *E. coli clpP* mutant was identical to the wild type at 37 °C and was only marginally affected when shifted to 45 °C or when 5% NaCl was added (Fig. 4). However, when the mutant was shifted to low pH we reproducibly obtained results showing that the growth was impaired compared to the wild type (Fig. 4) suggesting that ClpP in *E. coli* might have a particular function at low pH.

ClpP participates in proteolysis of misfolded protein

In previous studies it has been shown that ClpP is important for degradation of misfolded proteins in the Gram-positive bacteria *B. subtilis*; *L. monocytogenes* and *L. lactis*, whereas degradation of such proteins in *E. coli* is essentially unaffected by a *clpP* mutation (Frees & Ingmer, 1999; Gaillot *et al*., 2000; Kruger *et al*., 2000; Maurizi *et al*., 1990b). With the aim of investigating the turn-over of misfolded protein in the *S. typhimurium clpP* mutant both mutant and wild type cells were grown in the presence of the t-RNA analogue puromycin, which interferes with translation resulting in the production of misfolded puromycyl-containing peptides. Interestingly, we observed a decrease in colony size of the *clpP* mutant compared to the wild type suggesting that the *clpP* mutant is more sensitive to puromycin (data not shown). To examine whether a mutation in the *clpP* gene affects cellular proteolysis in *S. typhimurium*, the rate of degradation of puromycyl-containing polypeptides in LT1100 and wild type cells was determined as described by Raina and Georgopoulos (1990). The result, presented in Fig. 5, shows that the *clpP* mutant degrades these peptides both at a reduced rate and to a lower extent than the wild type, suggesting that ClpP is involved in the over-all degradation of misfolded proteins in *S typhimurium.*

Protein expression in *S. typhimurium clpP* mutant

With the aim of evaluating the impact of the *clpP* mutation on the accumulation of specific protein substrates during stress we analyzed protein expression at 45 °C of wild type and *clpP* mutant cells by two-dimensional (2D) protein gel electrophoresis. Since the deletion of *clpP* increases the cellular concentration of RpoS (Fig. 3) and the expression of a great number of genes is augmented by RpoS, we included the *clpP,rpoS* double mutant LT1104 in the experiment. By analyzing protein gels obtained with each of the three strains we found that the exposure of cells to 45 °C resulted in the enhanced synthesis of at least 10 proteins in the *clpP* mutant when compared to the wild type (Fig. 6 (b), boxes), whereas the synthesis of at least 6 proteins was decreased (Fig. 6 (a), boxes). Expression of five of these proteins is RpoS-dependent, since they were absent in the *clpP,rpoS* mutant (LT1104, Fig. 6 (b), circles). Of the remaining 11 proteins the amount of 5 proteins was increased in the absence of ClpP suggesting that they could be specific proteins substrates.

Discussion

Salmonella typhimurium is a facultative intracellular pathogen that during its infection cycle is exposed to hostile conditions in the host organism, including starvation, low pH, chemical and enzymatic stresses as well as elevated temperature (Foster & Spector, 1995). Recently, it was found that the proteolytic subunit, ClpP of the ATP dependent Clp protease complex is required for virulence of *S. typhimurium* both in a mouse model system and in macrophages (Hensel *et al*., 1995; Yamamoto *et al*., 2001; Webb *et al*., 1999). These findings prompted us to undertake a study of the physiological role of *clpP* in *S. typhimurium* stress response.

Components of the Clp protease complexes are found well conserved in prokaryotic cells (Schirmer *et al.*, 1996; Porankiewicz *et al*., 1999). In Gram-positive bacteria several studies have revealed that ClpP is important for surviving stress exposure (Frees & Ingmer, 1999; Gaillot *et al*., 2000; Msadek *et al*., 1998), whereas an *E. coli clpP* mutant was reported to be phenotypically identical to the wild type under various growth conditions (Maurizi *et al*., 1990b). When we analyzed a *S. typhimurium clpP* deletion mutant we found that, in contrast to wild type cells, growth of the mutant was impaired at low pH; at 45 °C and in the presence of 5% NaCl. Furthermore the mutant had a reduced ability to form colonies at the latter two conditions confirming its importance during stress exposure. Based on these results we investigated the mutants ability to degrade puromycyl-containing polypeptides which are truncated peptide products that are generated in the presence of the tRNA analogue, puromycin. We found that the turnover rates for these peptides were significantly decreased in the *S. typhimurium clpP* mutant compared to the wild type suggesting that the impaired growth of the mutant could be a result of the accumulation of misfolded proteins.

In addition to the degradation of misfolded proteins, *S. typhimurium* ClpP is likely also to be involved directly in the turnover of specific protein substrates. In agreement with previous findings (Webb *et al*., 1999) we observed that the stationary phase sigma factor, RpoS accumulates in the absence of *clpP* suggesting that it also in *Salmonella* is a target for the Clp protease. Since RpoS regulates or augments the expression of many stress-regulated genes in *S. typhimurium* and *E. coli* (O'Neal *et*

al., 1994; Loewen & Hengge-Aronis, 1994; Hengge-Aronis, 1996; Ibanez-Ruiz *et al.*, 2000) we questioned if the altered expression of one of these products was responsible for the stress sensitivity observed in the *clpP* mutant. To address this question we compared growth of an *rpoS* mutant with the growth of a *clpP, rpoS* double mutant and found that both grew like wild-type cells at 37 °C, whereas an increase in temperature or a downshift in pH resulted in impaired growth of the double mutant when compared to the single mutant or the wild type. Since the growth of the *clpP, rpoS* mutant was less affected than the *clpP* single mutant we conclude that ClpP affects growth in stress-exposed cells through both RpoS-dependent and -independent mechanisms.

To further investigate the accumulation of proteins in the *S. typhimurium clpP* mutant exposed to stress, we examined the protein expression pattern of wild type, *clpP* mutant as well as *clpP, rpoS* double mutant cells by two dimensional protein gel electrophoresis. By comparing protein expression at 45 °C we found that the *clpP* deletion altered the expression of 16 proteins of which 5 were regulated additionally by RpoS. Of the remaining 11 proteins the accumulation of 5 proteins was increased in the absence of *clpP* suggesting that they are putative ClpP substrates.

In *E. coli* the Clp protease degrades intrinsically unfolded protein substrates such as the CRAG protein (Kandror *et al*., 1999) and a non-secreted alkaline phosphatase mutant protein (Huang *et al.*, 2001) indicating that the proteins formed during stress could be degraded by Clp. The results we obtained with *Salmonella* therefore prompted us to analyze how an *E. coli clpP* mutant behaved when exposed to stress using the same experimental conditions as for *S. typhimurium*. In agreement with a previous finding (Maurizi *et al*., 1990b), we found that growth of the *E. coli clpP* mutant was only marginally affected by high temperature or addition of 5% NaCl. However, we did observe that low pH significantly affected growth of the mutant when cells were shifted from neutral pH to pH 4.5 suggesting that *E. coli* ClpP might play a particular role at low pH. Thus, our results indicate that the *S. typhimurium clpP* mutant is generally more sensitive to stress than the *E. coli clpP* mutant. In the literature it has furthermore been reported that the phenotype of an *E. coli clpP, lon* double mutant resembles that of a strain carrying a mutation in *lon* (Maurizi *et al*.,

1990b), the major ATP-dependent protease responsible for degradation of proteins generated during stress in *E. coli* (Chung & Goldberg, 1981; Maurizi *et al*., 1985). In contrast, the *S. typhimurium* the *clpP,lon* double mutant grows poorly (Wang *et al*., 1999) although the phenotype of a *S. typhimurium lon* mutant is similar to that observed in *E. coli* (Downs *et al.*, 1986). These observations further support the notion that ClpP has a more prominent role in degrading misfolded proteins accumulating during stress in *S. typhimurium* than in *E. coli.*

Acknowledgments

We would like to thank G.J. Phillips for supplying the plasmid pTSA29 and A. Matin for the strains AMS6 and AMS6P. Additionally we would like to thank C. G. Buerholt and D. O. Jensen for expert technical assistance. This work was funded by The Danish Research Council and the Centre for Advanced Food Studies.

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TABLE 1. Bacterial strains used in this study

Strain	Genotype	Source
***S. typhimurium* strains**		
JF2690	UK1 rpoS::Ap	(Lee *et al.*, 1995)
JF3717	UK1 xba-6014::Tn10dCm (48% linked to *clpP*$^+$)	(J. W. Foster, unpublished data)
JF3487	UK1 *clpP*1::Tn10dTc	(Webb *et al*., 1999)
C5	virulent wild-type	(Hormaeche, 1979)
LT1100	C5 Δ*clpP*$^-$	This work
LT1102	LT1100 with Tn10 linked to *clpP*$^+$ (linkage 48%)	JF3717 × LT1100, this work
LT1104	LT1100 *rpoS*::Ap	JF2690 × LT1100, this work
LT1108	LT1102 *rpoS*::Ap	JF2690 × LT1102, this work
LT1115	C5 *clpP*1::Tn10dTc	JF3487 × C5
KP1274	LT2 *(metA22 metE55 val galE*496 *rpsL*120 *xyl*-404 *H*1-*b nml*$^-$ *H*2-*e n x hsdL*6 *hsdSA*29 (r-m+))	(Enomoto & Stocker, 1974)
***E. coli* strains**		
DH5α	F$^-$φ80d*lacZ*ΔM15Δ(*lacZYA*-*argF*)U169 *deoR recA*1 *endA*1 *hsdR*17($r_K^-m_K^+$) *supE*44 *thi*-1 *gyrA*96	(Hanahan, 1985)
AMS6	K-12 (λ$^-$ F$^-$ Δ*lac*)	(Schultz *et al*., 1988)
AMS6P	AMS6 but *clpP*; Cam^r	(Schweder *et al*., 1996)

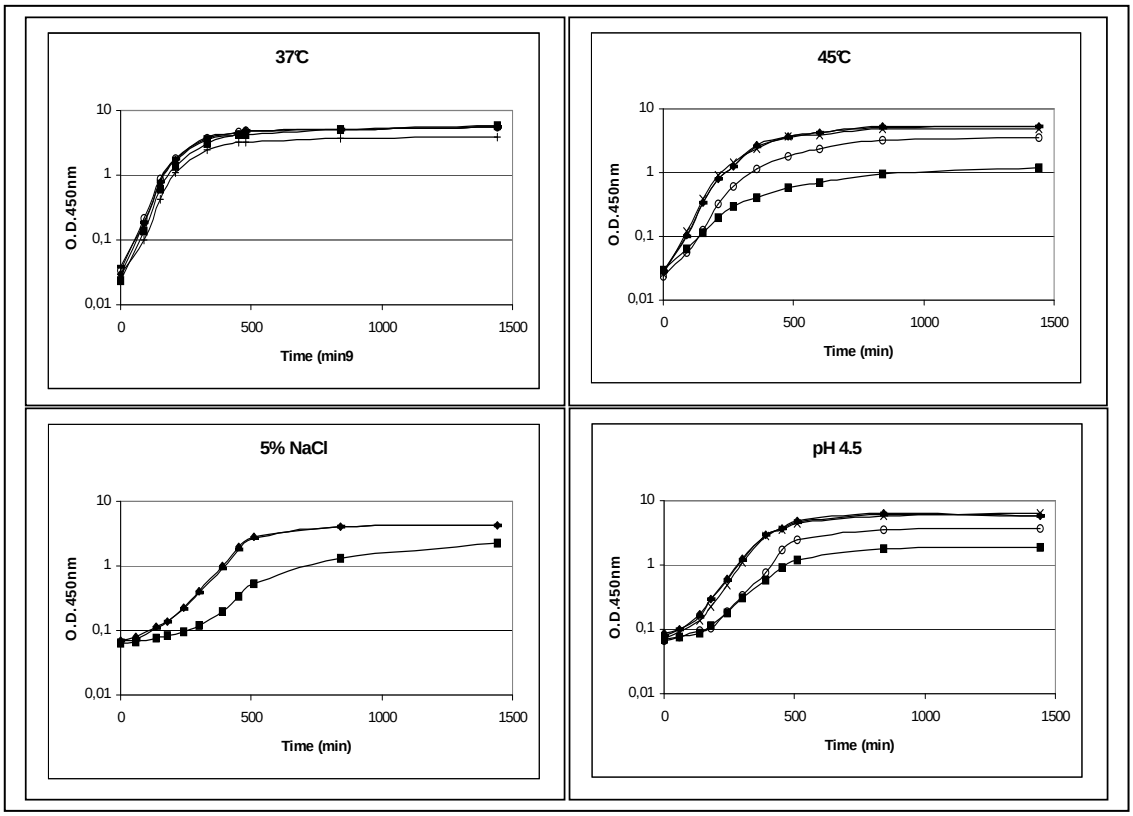

Fig. 1. LT1100 exhibits slower growth when exposed to stress.

Over night cultures were diluted into fresh medium and growth was followed at 37 °C; 45 °C; at pH 4.5 at 37 °C or in the presence of 5% NaCl at 37 °C. Diamonds: *S. typhimurium* wild-type C5, Squares: LT1100 (*S.typhimurium clpP*), Open circles: LT1104 (*S.typhimurium clpP*; *rpoS*); Crosses: LT1108 (*S.typhimurium rpoS*); LT1102 (*clpP*$^{+}$). The data shown are from one of at least three experiments that gave similar results.

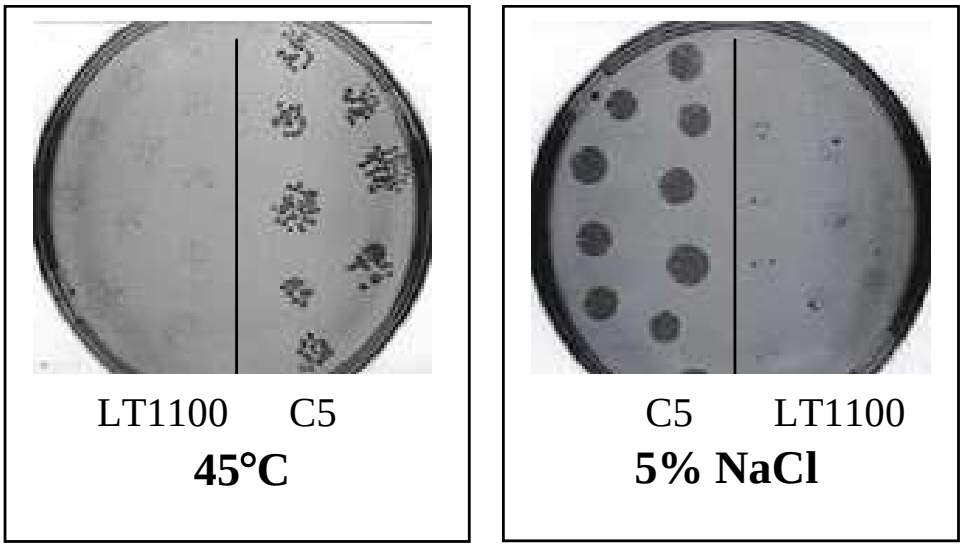

Fig. 2. The *clpP* mutant has reduced ability to form colonies under stress. Wild type *S. typhimurium* C5 and LT1100 (*clpP*) cells were grown exponentially and plated either at 45 °C (a) or on plates containing 5% NaCl at 37 °C (b). Growth was inspected after over night incubation.

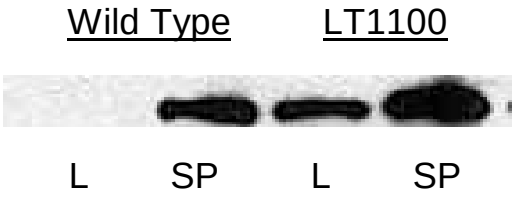

Fig. 3. Cellular σ^S levels in wild type and LT1100 cells.
Extracts were obtained from *S. typhimurium* C5 (wild type) and LT1100 (*clpP*) cells grown to mid-exponential phase (L) or late stationary phase (SP) and separated on SDS- PAGE protein gel. Protein samples were subjected to immunoblot analysis using antibodies raised against RpoS.

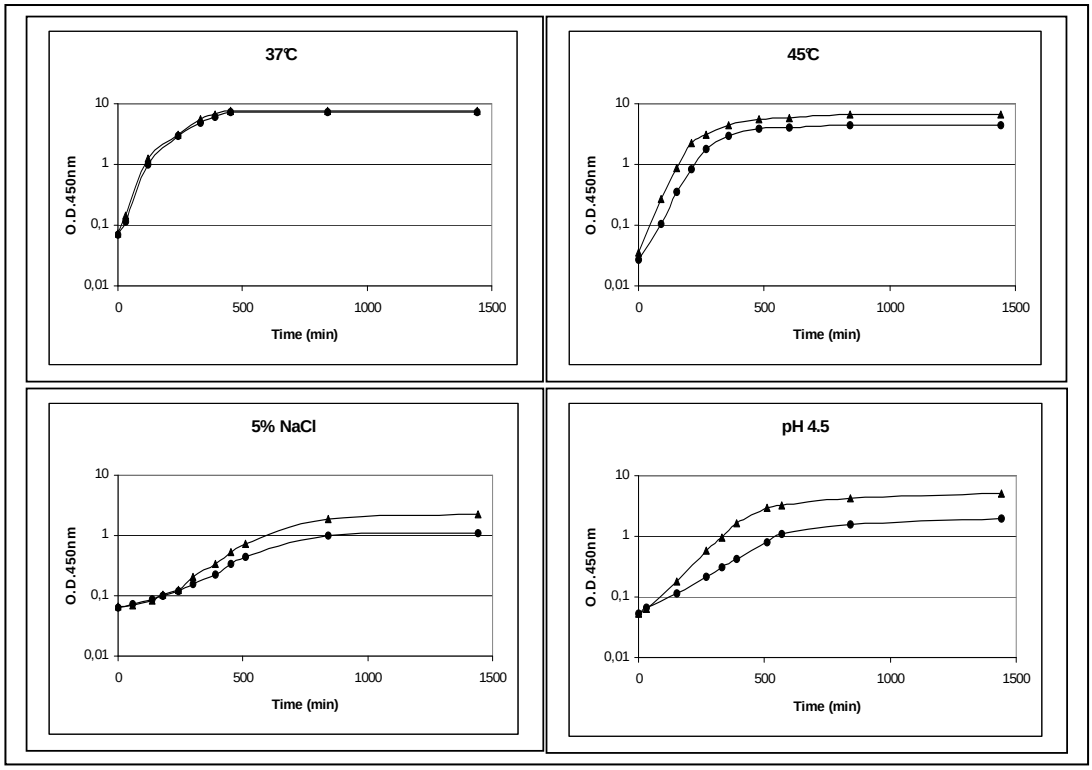

Fig. 4. Growth of the *E. coli clpP* mutant is impaired at low pH.
Over night cultures were diluted into fresh medium and growth was followed at 37 °C; 45 °C; at pH 4.5 at 37 °C or in the presence of 5% NaCl at 37 °C. Triangles: *E. coli* wild-type AMS6, Closed circles: AMS6P (*E. coli clpP*). The data shown are from one of at least three experiments that gave similar results.

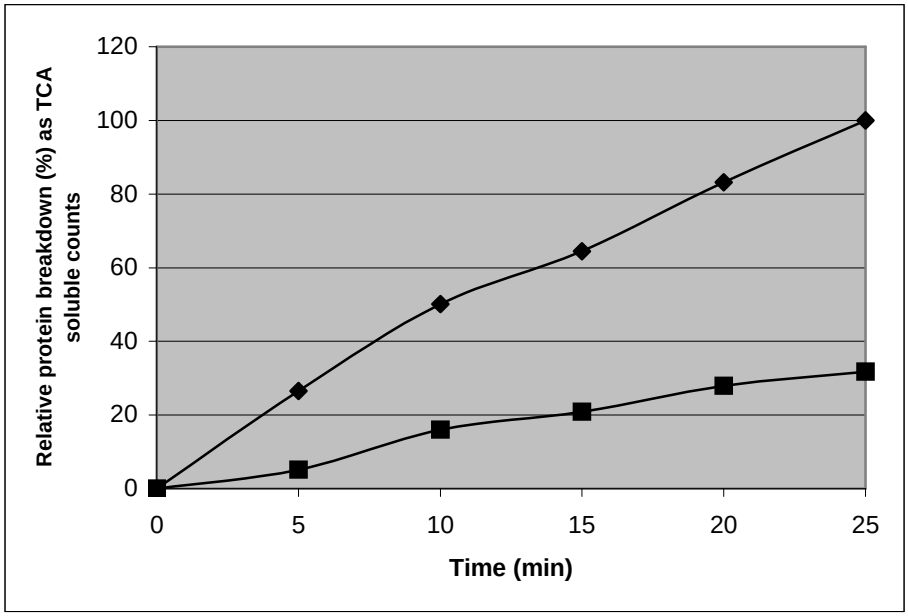

Fig. 5. The *clpP* mutant exhibits reduced proteolysis of puromycyl-containing polypeptides.

LT1100 and wild type cells were grown exponentially at 37 °C and following addition of puromycin cellular proteins were puls-labeled with [^{35}S]-methionine. Samples were taken at 5 min intervals and the relative protein breakdown (%) was determined as TCA-soluble counts relative to the TCA-soluble counts obtained for wild type cells 25 min after the chase. Diamonds: wild-type C5, squares: LT1100 (*clpP*). The data shown are from one of three experiments, which gave similar results.

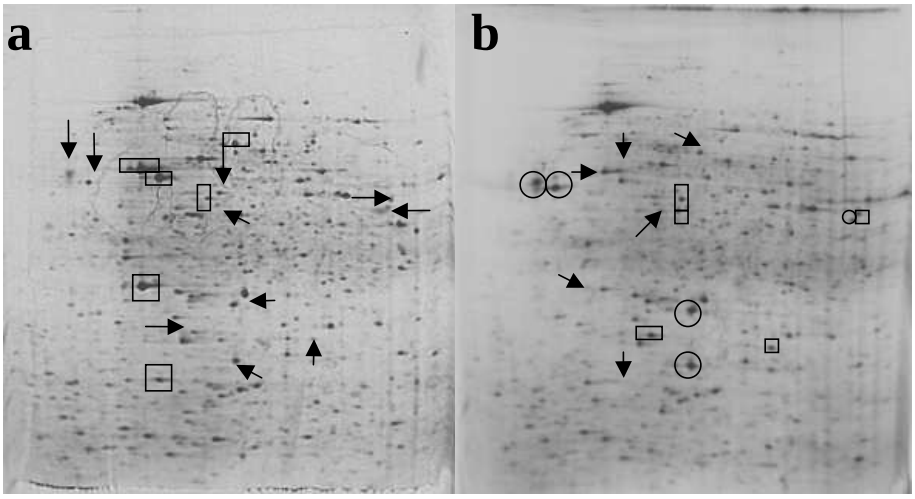

Fig. 6. Protein expression in the *clpP* mutant at 45°C.

Wild type C5 (a), LT1100 (b) and LT1104 were grown exponentially at 37 °C in M63 medium supplemented with casamino acids and shifted to 45 °C for 1 hour. Cells were puls-labeled with [^{35}S]-methionine. Proteins marked with a box on one panel and arrows on the opposite panel are those affected by *clpP*. A box indicates that a protein is produced in one strain but not in the other, where the corresponding location is marked by an arrow. A circle indicates a protein increased in LT1100 (*clpP*) when compared to wild type (marked with arrow), but is absent in LT1104 (*clpP*, *rpoS*).

Appendix C.

F 5

Statement of authorship

With reference to the Ministerial Order on the degree of D.Sc. (§ 5, stk. 4), it is required that, if the thesis or parts of it are the result of collaboration, statements of authorship are enclosed with the thesis at the time of submission. These should be signed by all authors and indicate the contribution of the candidate to the paper concerned.

With reference to the Ministerial Order on the degree of Ph.D. (§ 2, stk. 3), it is required that, if the thesis includes papers which are the result of collaboration, statements of authorship are enclosed with the thesis at the time of submission. These should be signed by all authors and indicate the contribution of the candidate to the paper concerned.

The supervisor can be a coauthor if the requirements of the Vancouver rules are fulfilled (see note).

This statement of authorship covers the following article: **"ClpP is involved in the stress response and degradation of misfolded proteins in *Salmonella enterica* serovar Typhimurium."**

***Salmonella* genes required for virulence and stress response. Characterization of ClpP and RfbM.**

which contributes to the following thesis: _____

submitted for the degree of D.Sc. / Ph.D. (underlined as appropriate)

The extent of **Line Elnif Thomsen**'s contribution
(doctor candidate / Ph.D.-student's name)

to the article is evaluated according to the following scale:

A. has contributed to collaboration (0-33%).
B. has contributed significantly (34-66%).
C. has essentially performed this study independently (67-100%).

Declaration for the individual elements	Extent (A,B,C)
1. Design of the project including formulation of problems to be tested and design of individual experiments.	B
2. Planning of experiments and design of methods to answer the problems posed under # 1 including choice and development of methods.	C
3. Involvement in analysis and experiments.	C
4. Presentation and interpretation and discussion of the results	C

F 5

Co-authors signatures			
Date	Name	Title	Signature
09.01.02	Hanne Ingmer	Associate Professor	
8/1-02	John E. Olsen	Professor	
13.12.01	John W. Foster	Professor	

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